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Casein Micelles from Bovine Milk: Native Structure, Interactions, and Practical Applications of their Structural Modification

Raymundo Trejo
rtrejo1@utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Raymundo Trejo entitled "Casein Micelles from Bovine Milk: Native Structure, Interactions, and Practical Applications of their Structural Modification." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

Federico M. Harte, Major Professor

We have read this dissertation and recommend its acceptance:

Qixin Zhong, John R. Mount, Juan L. Jurat-Fuentes

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Casein Micelles from Bovine Milk: Native Structure, Interactions, and Applications of Structural Modifications

A Dissertation Presented for the
Doctor of Philosophy
Degree
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Raymundo Trejo
December 2012

DEDICATION

This is dedicated to my parents, my grandparents (I made it, *buelitos*!), and most importantly to my beautiful bride and son. I could never have done this without them.

"Promise me you will always remember:
you are braver than you believe,
stronger than you seem,
and smarter than you think"
Christopher Robin

"Live like no one else will, so later you can live like no one else can."
Dave Ramsey

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I would like to first give thanks to my parents; after all I would not be here without them. Thanks to my sister, niece, family, and in-laws; they are an important part of who I am. Thanks to my friend Victor Parra who has, for reasons unbeknownst to me, stood by me for most of my life. I have the deepest gratitude for the guidance and mentoring of Mr. Lawrence V. Löf and Mr. Guillermo Aguilar from the Rancho del Cielo Field Station Program at the University of Texas at Brownsville, I am who I am today in no small part because of them. Thanks to Dr. Federico Harte for taking me on as a student, and teaching me how to be a scientist. Thanks to Dr. Juan Jurat-Fuentes, Dr. Qixin Zhong, and Dr. John Mount for their expertise, guidance, and patience as part of my doctoral committee. Thanks to the faculty and staff of the Department of Food Science and Technology for their guidance, teaching and assistance. Thanks to the Graduate School and the UT Agricultural Experiment Station for their support. Thanks to all of the members of the Harte lab group both past and present: Adrianne Roach, Shan Xu, Vyku Ganesan, Aaron Hernandez, Songsong Li, Maneesha Mohan, Vinay Mannam, Virginia Artegoitia, Ran Ye, Sasha Wilkinson, and Manpreet Cheema for helping me along and keeping me on the right track. My sincere gratitude goes to Carrie Pratt, Eric Goan, and Nathan Miller for being my dearest of friends during graduate school. They never let me falter from my quest. To all the folks that walked beside me as my fellow graduate students, specially Minfeng Jin, Koi Techathuvanan, and Stella Chen, I give many thanks for all their friendship and encouragement. Above all… to Rosa, my best friend and my bride, and my son Victor Luis: I have no words to describe how much I value everything they bring into my life every single day.

ABSTRACT

A definitive structure of the native casein micelle structure continues to elude researchers. Data and images obtained via cryo - transmission electron microscopy of isolated native casein micelles allowed for the reconstruction of a three dimensional model of the micelle; which contains water filled cavities (*ca.* 20 to 30 nm diameter), channels (diameter larger than *ca.* 5 nm), and several hundred high density nanoclusters (6-12 nm diameter) within the interior of the micelles.

Analysis of isolated casein micelles by SDS polyacrylamide gel electrophoresis (pH 6.8, 5.0) showed that whey proteins were found associated with the isolated micelles regardless of the pH of the milk sample. However, the introduction of the hydrophobic peptide valinomycin (MW= 1111.32; LogP=5.92) into raw skim milk did not result on the peptide being found associated with isolated casein micelles.

Taking advantage of the heat, ethanol, and pH mediated micelle disassociation, it was possible to improve the solubility of caseins in fluid milk via esterification. The esterification of casein resulted in a shift on its isoelectric point; which in turn resulted in a better solubility at low pH values. Caseins esterified at a pH 3 and 4 had a higher solubility than those esterified at other pH values and non-modified skim milk powder in acidified juice with a pH equal or below 3.5.

By adding a chilling step (3-5 ^{o}C/pH ~5.2) to the processing of non-fat yogurt, it was possible to improve the whey holding capacity of the product while also producing a firm product without the need for added stabilizers or gums. In the conditions of the chilling step, beta casein and calcium migrated out of the micelles. This resulted in a reduction on the size of the micelles. A possible explanation for these results would be

that once the temperature returned to 47 ^{o}C, the liberated caseins self-associated into small micelles and together with the smaller original micelles formed a tight gel matrix.

Casein is fundamentally important to the dairy industry. By gaining an understanding of its structure and properties, it is possible to develop new applications and enhance its performance in dairy products.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I
INTRODUCTION & LITERATURE REVIEW

Introduction

Caseins are the primary protein components in bovine milk. These proteins form large colloidal particles of 50-600 nm in diameter, known as casein micelles. Many of the technologically important properties of milk, e.g., its white color, stability to heat or ethanol and coagulation by rennet, are due to the properties of the casein micelles. Therefore, there is an economical and technological incentive in characterizing their properties and determining their structure (Fox and Brodkorb, 2008). The following review will cover the most accepted models for the casein micelle structure and the applications for the casein micelle in the food industry and beyond.

Milk casein micelle structure

Casein makes up the majority of milk proteins in bovine milk (≈80%). Because of this fact, research involving caseins can be dated to the early nineteenth century. The term “casein micelle” was first utilized by Beau in 1921 (Fox and Brodkorb, 2008). Interest in the casein micelle has remained constant through the years, and research on the subject continues to be carried out throughout the world. The properties of the casein micelles are summarized in **Table 1.**

Table 1.1 *Average characteristics of casein micelles (from Fox et al., 2008)*

Characteristic	Value
Diameter	120 nm (range: 50– 500 nm)
Surface area	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g cm^3
Mass	2.2×10^{-15} g
Water content	63%
Hydration	3.7 g H_2O g^{-1} protein
Voluminosity	44 cm^3 g^{-1}
Molecular mass (hydrated)	1.3×10^9 Da
Molecular mass (dehydrated)	5×10^8 Da
No. of peptide chains	5×10^3
No. of particles per mL milk	$10^{14} – 10^{16}$
Surface of micelles per mL milk	5×10^4 cm^3
Mean free distance	240nm

The function of milk is to serve as a medium for the mother to provide nutrients and minerals to their offspring. The principal mineral present in milk is calcium. The dry matter of bovine casein micelles is ~ 94% protein and ~ 6% mineral, referred to collectively as colloidal calcium phosphate (CCP). This CCP is regarded as the cement which holds the micelle together, since its removal by the EDTA sequestration of calcium or by dialysis against a calcium phosphate free buffer results in dissolution of the micelles at lower temperatures (Horne, 2006). Due to the large amount of calcium present within the casein micelle it is well accepted that it serves as a mechanism for calcium transfer in mammals (Patton, 2004).

In their 2008 review of the importance of the casein micelle to the food industry, Fox and Brodkorb further illustrated that in good quality milk, the

casein micelles are stable to most processes to which milk it is normally subjected:

- In its concentration by evaporation or ultrafiltration, the stability of the micelles decreases with the increasing degree of concentration. This is due mainly to a closer packing of micelles, an increase in [Ca^{2+}], and a decrease in the pH value due to the precipitation of CaH_2PO_4 and $CaHPO_4$ as $Ca_3(PO_4)_2$, accompanied by the release of H+.
- In the dehydration of milk, unless there are heat-induced changes, the micelles in milk powder reconstitute readily with only minor changes in their properties.
- Freezing has little, if any, effect on the casein micelles. However, slow freezing and storage at temperatures ranging from -10 to -20 ˚C causes a destabilization of the micelles due to an increase in [Ca^{2+}] and a decrease in pH, due to the precipitation of $Ca_3(PO_4)_2$; these effects are intensified by the crystallization of lactose, which lowers the freezing point of milk.
- The normal homogenization of milk (up to 20 MPa) has little or no effect on the micelles. However, high-pressure homogenization (>200 MPa) or high-pressure treatment >200 MPa result in the dissociation of the micelles.
- High temperature short time (HTST) pasteurization (72˚C for 15 s) has little or no effect on the micelles, but heating at higher temperatures causes denaturation of the whey proteins and their interaction with the casein micelles via sulphydryl-disulphide interactions, especially between

β-lactoglobulin and κ-casein. Severe heating, especially of concentrated milk, causes Maillard browning, a decrease in pH, dissociation of κ-casein from the micelles, and coagulation. (Fox and Brodkorb, 2008).

The micelles can be destabilized by a number of factors, some of which are industrially important: hydrolysis of the κ-casein by selected proteinases, which is exploited in the manufacture of most cheese varieties; acidification to about pH 4.6, which is exploited in the manufacture of some cheeses, fermented milks and functional caseinate products; ethanol (or other alcohol); anionic detergents (e.g., SDS); high pressure (Fox and Brodkorb, 2008).

In spite of the large amount of research conducted, there is still not a definitive model for the casein micelle structure. Several models have been proposed based on the properties and behavior of the individual caseins and of the casein micelles. The most accepted structure models are the coated sphere model, the sub-micellar model, the Holt model and the Horne model.

The coated sphere models takes into consideration the properties of κ-casein, which is soluble at the calcium concentration in milk and which is about 12% of total casein, and it can stabilize about 10 times its mass of Ca-sensitive caseins (α_{s1}-, α_{s2}- and β-) (Fox and Brodkorb, 2008). In this model, the casein micelles are coated with a "hairy layer" of protein, mainly κ-casein. For this type of model the internal structure is not relevant, unless it changes in the course of processing. The coated sphere model is consistent with the observation that most of the κ-casein is on the micellar surface. It is also consistent with the fact that the micellar hydrodynamic diameter has been shown to decrease during

renneting in dilute suspensions. Since the action of chymosin removes the protruding macropeptide portion of the κ-casein molecule, the decrease in micellar diameter is consistent with the loss of the hairy layer (Dalgleish et al., 2004).

The κ-casein also plays a role in the sub-micelle model, where the individual caseins come together in their appropriate proportions to form internal sub-micelles.In this model the micelle is held together mostly by the presence of κ-casein. However, if the casein micelle is depleted of κ-casein, or lacks external sub-units rich in κ-casein, then the colloidal calcium phosphate is regarded as the cement which links these sub-units together. A graphical example of the sub-micelle model can be seen in **Figure 1.1**.

In the Holt model, which is illustrated in **Figure 1.2**, the micelle is regarded as a mineralized, cross-linked protein gel, the colloidal calcium phosphate nanoclusters are the agents responsible for cross-linking the proteins and holding the network together (Horne, 1998).

The formation of nanoclusters would drive micelle formation by randomly binding phosphoproteins causing an inverted micelle. More proteins could then coat this new hydrophobic surface and in turn, bind more calcium phosphate until a size limited colloid is formed. (Farrell et al., 2006).

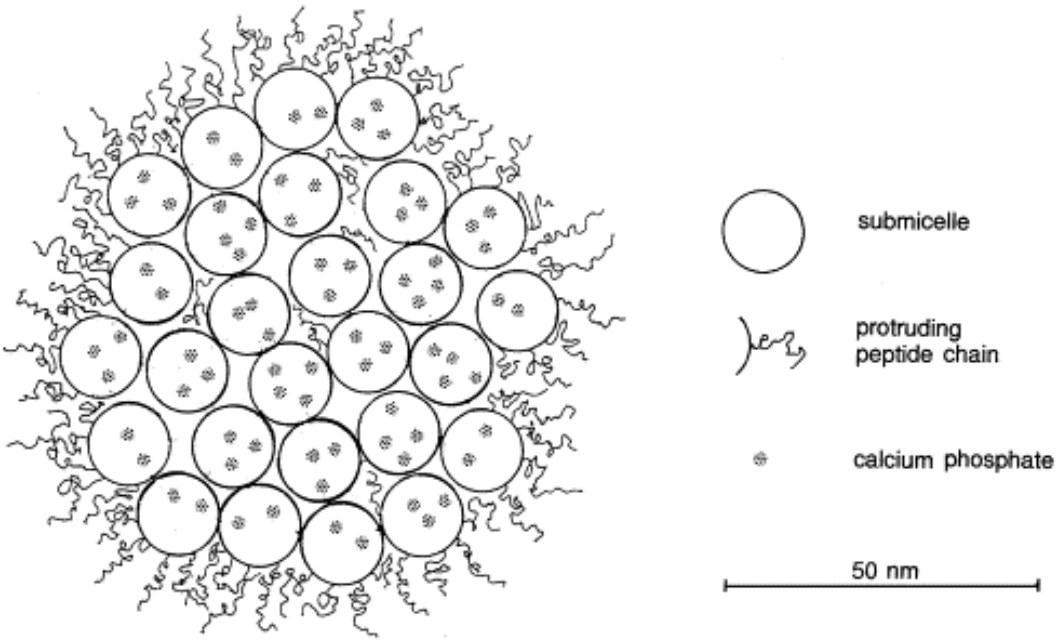

Figure 1.1 *Sub-micelle Model (cross section) of a casein micelle (From Walstra 1999)*

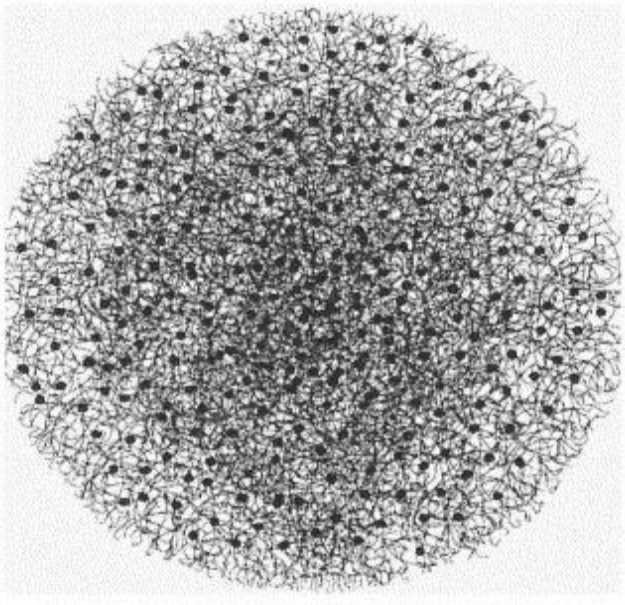

Figure 1.2*. The Holt model of casein micelle structure (From Farrell et al., 2006).*

The Horne model, also known as the dual-binding model (**Figure 1.3**), can be considered an extension of the Holt model. Horne, in contrast to Holt, considered the surface chemistry of the individual caseins and concluded that protein-protein interactions were indeed important, but in essence the model retains the gel concept. The amphiphilic nature of the caseins causes them to act more as block copolymers of alternating charge and hydrophobicity. Here again the growth of the calcium phosphate nanoclusters begins the process of micelle formation, but nanocluster growth is limited by binding to the phosphopeptide loop regions. Once bound to the amorphous inorganic matrix, further protein-protein interactions are related to the hydrophobic blocks and polymerization proceeds by repeating the entire process.

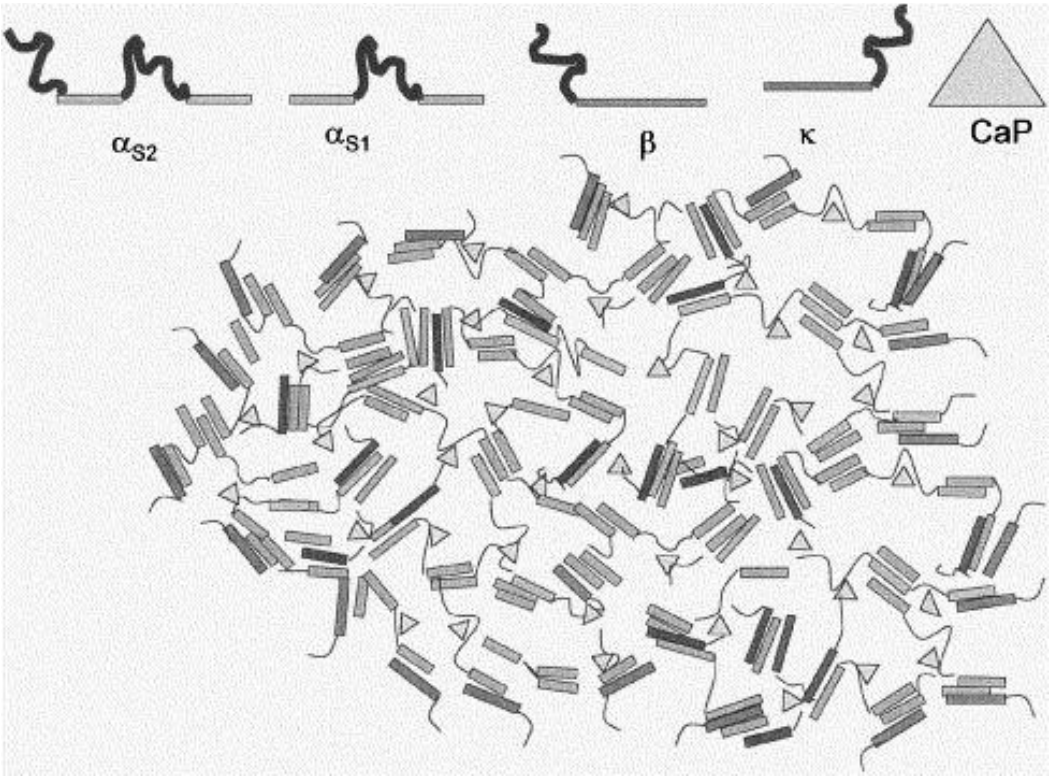

Figure 1.3. *The Horne model of the casein micelle (From Farrell et.al. 2006)*

Micelle formation leads to an internal gel-like structure with nanoclusters of calcium and phosphate embedded within that structure. Since they are the bounding sites for nanocluster formation, the numbers of phosphoserine residues present in the casein proteins influence the number of nanoclusters being formed. Therefore, the reaction of κ-casein which contains only one phosphoserine residue, limits micellar growth by acting as a dead end capping unit in analogy with the growth of synthetic polymers (Farrell et al., 2006).

The controversy and debate over the correct model of the casein micelle is continuously evolving, as new research uncovers further clues into the nature of the casein micelle. One such development involves the use of electron microscopy studies, from which Dalgleish *et al.* have proposed a possible new model for the bovine milk casein micelle. Their results seem to show that the micelle consists of tubules, presumably of caseins, the ends of which protrude from the bulk of the micelle structure. These tubules are about 20nm in diameter, which is consistent with the dimensions of the calcium phosphate/casein nanoclusters proposed by Holt (Holt et al., 1998). However, the micelle is still protected from close approach of large particles (such as other micelles) by the protruding tubules. On the other hand, there are large amount of spaces, where individual protein molecules, or even small aggregates of proteins, can approach the micelle (Dalgleish et al., 2004).

It is generally known that whey proteins, primarily β-lactoglobulin, interact with the casein micelles as a result of heat treatments. β-lactoglobulin attaches to the micellar κ-casein through disulphide links. Apparently, even

polymerized β-lactoglobulin can bind to the casein micelles in this way, so that the casein micelles may acquire new surfaces and therefore develop new properties, the change being dependent on the severity of the heat treatment (Dalgleish, 1993).

Based on current developments, Dalgleish postulates that water in the interior of the casein micelle is unlikely to be evenly distributed throughout the structure. The reason behind this would be the random internal architecture of the proteins based on the hydrophobic interactions. He proposes the existence of cavities and channels throughout the structure of the micelle (Dalgleish, 2011). An illustration of this proposed structure can be seen in **Figure 1.4**. A new model based on observations of cryo-transmission electron microscope images has been proposed by McMahon et al. In this model, known as the open lattice model (**figure 1.5**), the micelle has an irregular structure and the proteins have a large diversity of linkages among themselves. Like all the other models, the κ-caseins extend outwards of the main micelle structure. However, the number of these extensions is much smaller than has been previously postulated (McMahon and Oommen, 2008).

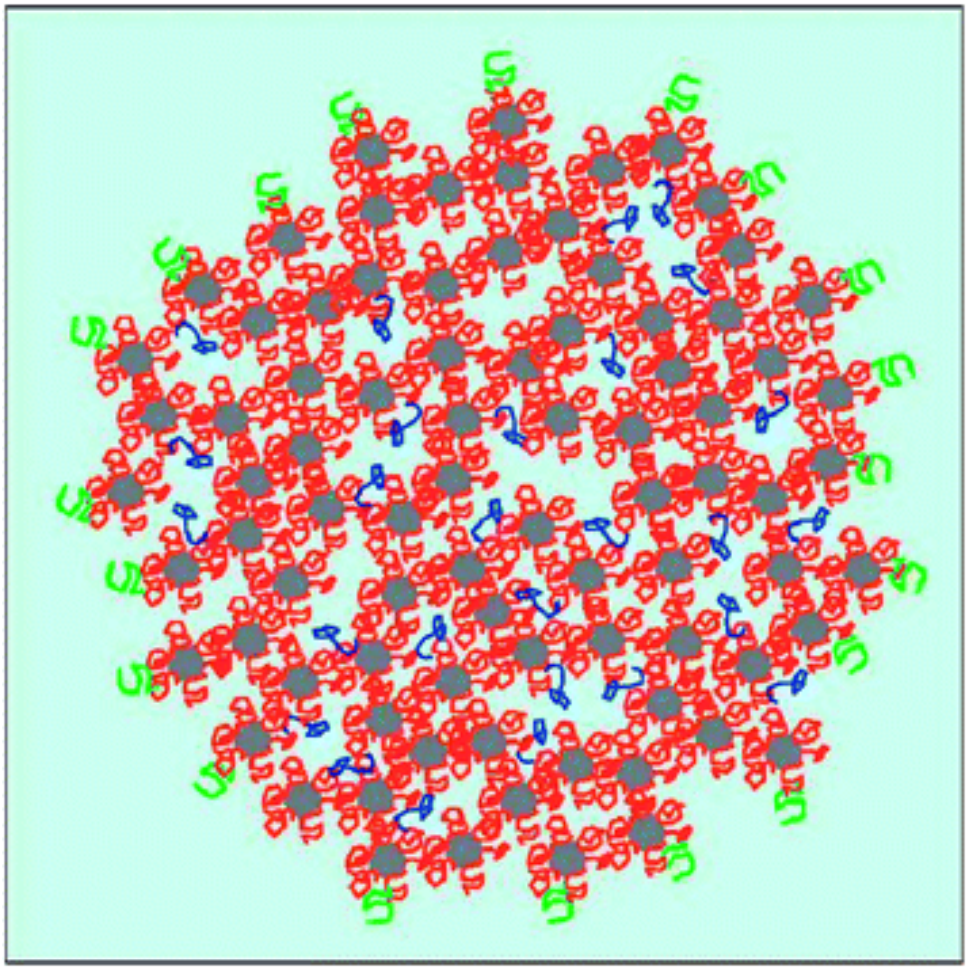

Figure 1.4. *Schematic structure of the casein micelle, incorporating calcium phosphate (grey) with their attached caseins (red) and the surface-located κ-casein (green) other caseins are shown in blue (from Dalglesih, 2011).*

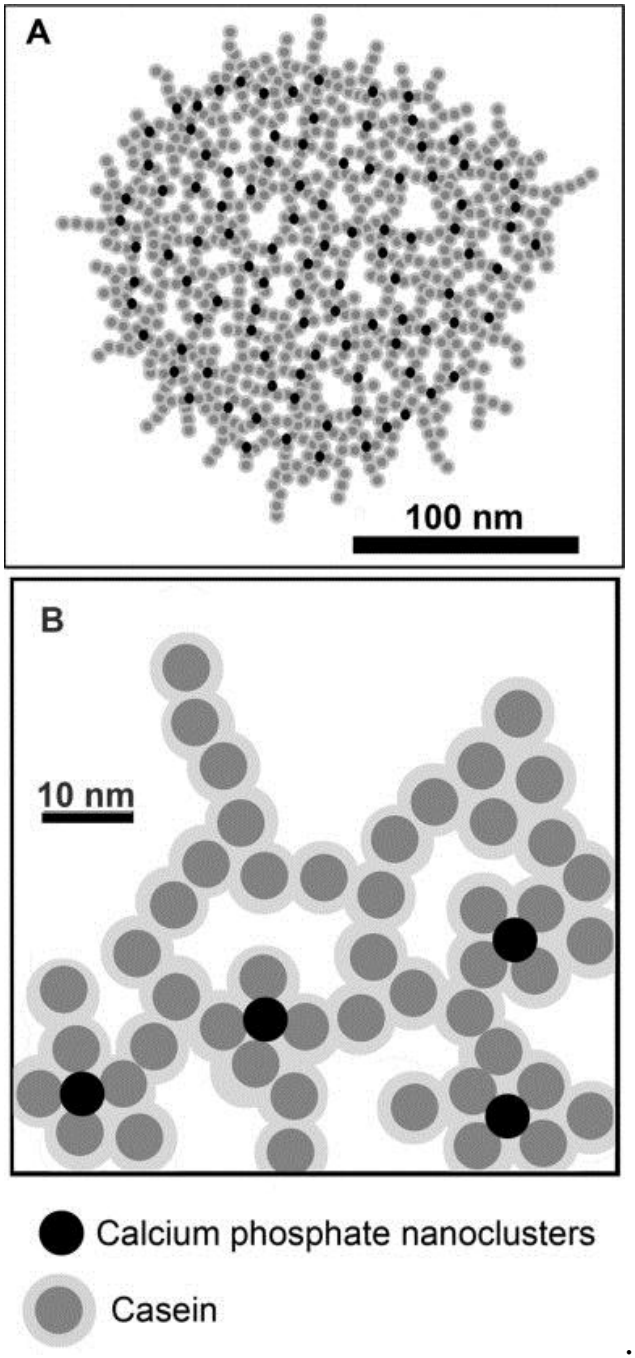

Figure 1.5. *Schematic diagram of the interlocking lattice model of the casein micelle with casein-calcium phosphate aggregates throughout the entire supramolecule and chains of proteins extending between them (from McMahon and Oommen, 2008).*

Food Ingredient & Colloidal Applications of the Casein Micelle

As mammals, humans are able to survive exclusively on milk during early infancy. Milk serves as a medium for the mother to provide her infant with both nutrition and immunity. Later in life, during childhood and beyond, milk can provide high nutritional value to the daily diet. Therefore, bovine milk is widely accepted as a food source throughout the world. There are a large variety of dairy products; however, most of them are made using a relatively small number of processes. These include heating (at temperatures between 60 and 140 °C), concentration (by evaporation or ultrafiltration), homogenization, fermentation, and treatment with enzymes. These operations may be combined. In addition, milk may be separated, dried and recombined, and milk components may be used as ingredients in other foods (Dalgleish, 1993).

Milk proteins are one of these components which are widely used as food ingredients. The popularity of milk proteins as food ingredients is due to several reasons. As well as being nutritious, milk proteins exhibit a range of important functional properties. These include gel, curd, emulsifying, foaming, ion-binding and flavor-binding; all of these properties are different for the various proteins (Dalgleish, 1993). Traditional milk protein products include: nonfat dry milk powder, whey powder, whey protein concentrate, whey protein isolates, caseins, and caseinates.

As such, proteins make up a very small portion of the components in milk. Bovine milk contains generally between 3 and 3.5% by weight of true protein, the

amount depending on the breed and individual variation of the animal and, to a lesser extent, on the stage of lactation, the nutritional status, and the health of the animal (Dalgleish, 1993). For example, nonfat dry skim milk is prepared by separating the milk fat from the milk, usually by centrifugation, and then spray-drying the skim milk. Therefore, this product contains all of the proteins found in milk. Among the milk proteins, casein is the most abundant protein. Caseins represent ≈ 80% of the total protein in the milk of cattle and other commercial dairying species (Fox and Brodkorb, 2008). Casein isolates are usually prepared by isoelectric precipitation at pH 4.6. This method was developed by Hammersten (1883) and improved by Van Slyke and Barker (1918). Caseinates are obtained by treating the rennet or acid casein with an alkali (sodium or calcium), which is then dried. The remaining proteins found in milk are found in the milk serum or whey (i.e., the solution which is left when the casein micelles and the fat globules are removed from milk). These proteins remain soluble when the casein precipitates at the isoelectric point of 4.6. Whey contains four major proteins, namely β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins (Dalgleish, 1993).

Whey powders are commonly used as ingredients in the food industry. They are prepared by separating the fat from the whey produced during cheese making, concentrating the whey to 40-50% solids, and then spray drying to produce a powder. The most common commercial method to produce whey protein concentrate from the skimmed whey solids uses ultrafiltration to separate the protein fractions. Then the fractions are condensed via evaporation and spray

dried to produce a powder. These concentrates contain all of the whey proteins; however uses for the individual proteins are currently being researched.

α-lactalbumin is present at higher levels in human milk than in bovine milk and therefore the fortification of infant formula with respect to this protein is thought to improve its comparability to human milk. Hence, at present, the main interest of the food industry in this protein appears to be infant formula applications (Playne et al., 2003). β-lactoglubulin is the principal whey protein. As a member of the lipocalin family, biological functions of β-lactoglobulin have been associated with the binding and carrier functions of small hydrophobic molecules in the hydrophobic cleft. For use as an emulsifier, the surface activity of β-lactoglobulin is very dependent on the pH of the system. At low pH values, the protein takes on a more compact and rigid conformation. This would lower its performance as an emulsifier. Increasing the pH of the system from 3 to 7 allows for the protein to more readily rearrange at the interface. This results in an improvement of the protein's emulsifying properties (Wong et al.,1996). Currently, the main use of the whey proteins is to protein fortification of food products. Unlike the casein micelle, the whey proteins are stable to pH changes, allowing for their use in acidified beverages. However, whey proteins are unstable to heat treatment resulting in gel formation depending on the pH of the sample (Wong et al.,1996). Future opportunities for developing the useful functional properties of β-lactoglobulin as a functional food ingredient for humans may lie in the development of its carrier function in either aggregated or soluble forms (Playne et al., 2003). It is important to mention that whey proteins, and β-

lactoglobulin in particular, are the principal allergen component in milk. Allergic reactions to casein are less frequent to whey proteins (Monaci et al., 2006). These allergenic properties could limit their use on some products.

Milk proteins can be also used as "functional" food ingredients. Functional foods (sometimes referred to as physiologically functional foods, nutraceuticals, designer foods or pharmafoods) can be defined as those that provide the consumer with an identified health benefit above and over basic nutritional value (Playne et al., 2003). One example of a milk protein serving as a functional food ingredient makes use of the calcium carrier function of the caseins. This food ingredient has been developed by proteolysis and the extraction of the casein phosphopeptide (CPP) class of peptides. The product is commercially available as the food ingredient 'Recaldent™', which contains CPP. This product has been demonstrated to protect against the demineralization of tooth enamel and its application in various oral care and food products is in progress (Playne et al., 2003).

Another possible practical application for casein includes acting as a fat replacement in dairy products. The performance of acidified sodium caseinate emulsion foams containing oil versus whipped cream was evaluated by Allen et al. (2006). The foam volume, stability and rheology in the casein stabilized foams were determined to be dependent on the pH values and the concentration of calcium ions. The foams were stabilized by the aggregation of the protein coat surrounding the oil droplets. The foams that were formed at low pH were more stable than whipped cream while the foams formed at high pH were liquid like

and unstable. This instability was mostly due to syneresis in the foam (Allen et al., 2006). Allen et al also studied the effects of adding hydrocolloids to the foam as well as using oils and fats in the foam. They found that replacing all of the liquid oil droplets for solid droplets led to a considerable increase in the elastic modulus of the foam behaving very similarly to whipped cream. The addition of a hydrocolloid, in this case pectin, to both liquid droplet and solid droplet containing systems resulted in an increase to both the elastic modulus and the yield strain. Therefore, these foams were closer to whipped cream in terms of foam stiffness, but less similar in terms of their large deformation rheology (Allen et al., 2008). The practical applications for casein and the casein micelle are not limited to the food industry. New strategies are being explored to utilize casein in the pharmacology industry.

One such application that can be used in the food and pharmaceutical industries is that of casein nanogel particles. Huppertz et al. investigated the structure and stability of casein nanogel particles created by cross-linking caseins within the casein micelle with transglutaminase. The particles consisted of a covalently linked network from which the micellar calcium phosphate could be removed without having a major effect on the structure stability. The behavior of the nanogel particles was similar to that of native casein micelles when studied via neutron diffraction, x-ray diffraction, and light scattering. This indicates that the particles have a size and structure similar to the native micelles. However, when compared to native micelles, the nanogel particles were more stable to heat induced coagulation, and less stable to acid induced coagulation. Modifying the

amount of micellar calcium phosphate of the nanoparticles from 0% to 150% of its original concentration strongly affected the colloidal stability of the particles. The stability to both heat and acid induced coagulation increased with a decreasing micellar calcium phosphate content (Huppertz and de Kruif, 2008). Both the food industry and the pharmaceutical industry have shown an interest in harnessing the carrying and binding properties of the casein micelle for encapsulation and delivery of compounds. In **Figure 1.6**, Livney illustrated the mechanisms and properties of milk proteins that can be utilized for delivery tasks (Livney, 2010).

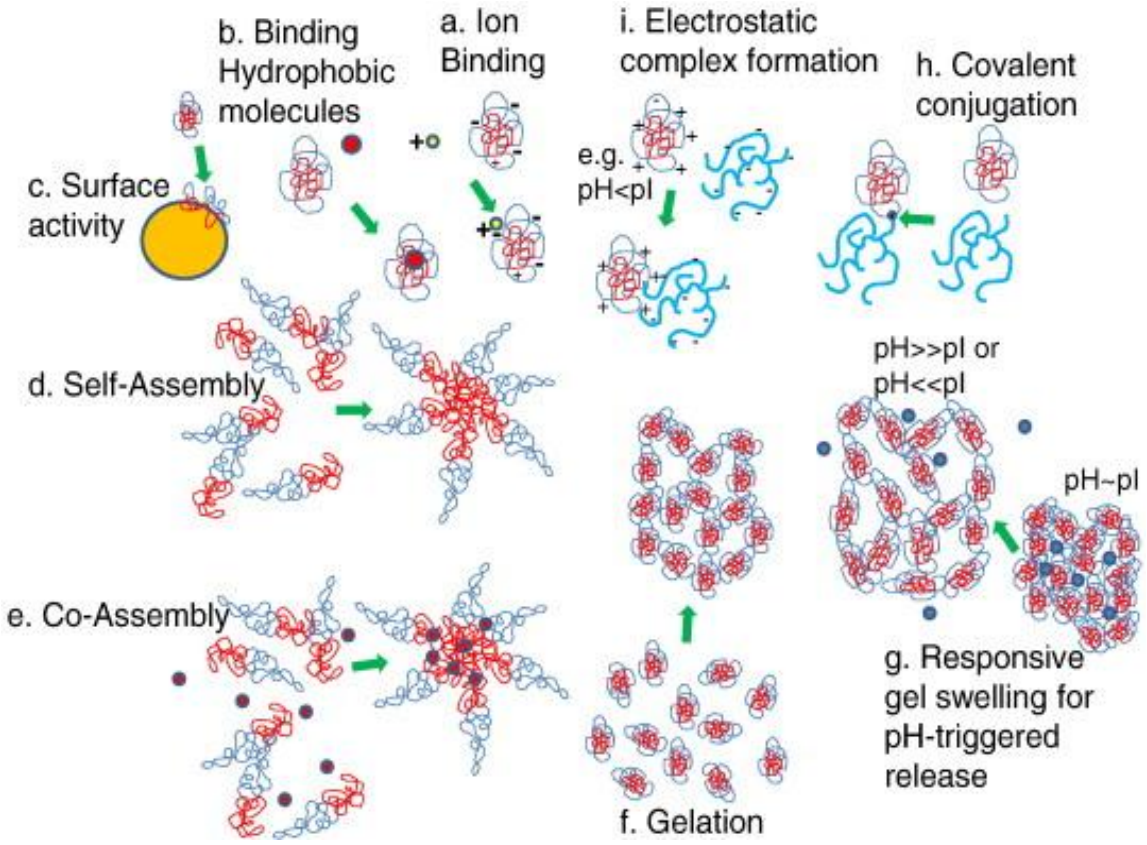

Figure 1.6. *Illustrations of several functionalities of milk proteins which are useful for delivery tasks (from Livney, 2010)*

The self-assembly property of the casein proteins has been utilized to encapsulate materials within the micelle. This approach was used by Sahu et al. to encapsulate curcumin, a flavor compound that has anticancer properties, with micellar casein. The reformed micelles had an average size distribution of < 200 nm. The curcumin bound to the casein micelles, possibly via hydrophobic interactions (Sahu et al., 2008). This property was also utilized by Zimet et al. to protect and deliver ω-3 polyunsaturated fatty acids. In their work, they encapsulated docosahexaenoic acid (DHA) with reformed casein micelles. The casein micelles protected the DHA against oxidation and had a good colloidal stability when the product was kept at 4 ^{o}C (Zimet et al., 2011).

The gelation properties of casein were utilized by Heidebach et al., to encapsulate probiotic bacteria in order to shield them from the acid conditions in the stomach and provide a better means of delivery to the intestines. They encapsulated the bacteria with a cold-set gelation by rennet following water-in-oil emulsification. They attributed the protection of the bacteria in the low pH conditions to the buffering capacity of the milk proteins (Heidebach et al., 2009). The responsive gel swelling for a pH driven release was shown by Song et al. to lower the release of bovine serum albumin (BSA) in a low pH environment similar to gastric conditions. However, upon a rise in the pH that simulated the conditions in the intestines the gel swelled and released the BSA into the medium (Song et al., 2009). This method would also be useful as a method for delivery of probiotics, live beneficial microorganisms, to the intestines without a loss during gastric digestion.

Conjugates of casein and whey proteins were suggested by Ye et al. as a means of encapsulating fish oil for the enrichment of cheese. The fish oil was encapsulated with a surface layer of preheated milk protein complexes. This resulted on a stable emulsion with an average droplet size of 0.21 μm. The encapsulation of the fish oil resulted in a lower oil oxidation than the oxidation observed with non-encapsulated fish oil. The cheese also had a higher sensory quality than the cheese containing non-encapsulated oil. The results of a trained panel showed that processed cheese containing encapsulated fish oil could have a higher concentration of fish oil without any negative off- flavor being perceived. (Ye et al., 2009). Nielsen et al. demonstrated that caseinate serve as a good barrier against oxidation when it was used as an emulsifier for fish oil. The caseinate showed a similar level of protection as the levels achieved by modified atmosphere packaging. They attributed the good performance to a physical barrier effect, and possible chelation of oxidizing metal ions (Nielsen and Jacobsen, 2009). It has also been shown by Semo et al. that caseins could also serve as a shield against radiation. Caseins have strong UV absorbance properties, around λ = 200-300 nm, which can be used to protect light sensitive compounds. Semo et al. demonstrated that reformed casein micelles provide a significant protection against deterioration for encapsulated vitamin D (Semo et al., 2007).

Besides being used as carrier vehicles, the use of casein to fabricate films for coating of pharmaceuticals has been explored (Abu Diak et al., 2007). The film prevented a significant release of medicine in a pH similar to the one found

in the stomach, but had a significantly larger release of medicine in a pH similar to intestinal conditions. In the stomach, the casein micelles are also subjected to pepsin digestion. This digestion process results in the aggregation of the micelles into a coagulum prior to moving into the intestines (Qi et al., 2007). This results in a loss of proteins, but would protect any compound or protein encapsulated within the micelle from the enzymatic action. This process takes advantage of the native function of the micelles as a means for calcium delivery to the infant. The micelles form a coagulum in the stomach, which protects the calcium and insures its delivery into the intestines for uptake. The electrostatic complexation of pectin and caseinate was investigated by Rediguieri et al. The reduction of the pH of a caseinate solution below the isoelectric point of casein (≈4.6) results in the proteins having a negative charge. This in turn attracts the negatively charged pectin (pKa ≈ 3). The resulting complex produced micro-particles averaging 3µm in diameter in a reversible process. As the pH increased, the pectin molecules migrated to the continuous phase and the particles became soluble (Rediguieri et al., 2007).

Conclusion

Several models, such as the sub-micelle, open lattice, and nano-cluster, for the structure of the casein micelle have been proposed. There are similarities between the models; however, authors disagree on the arrangement of the internal architecture as well as the driving force behind micelle formation.

By taking advantage of the properties of the casein micelle, it is possible to utilize it as a delivery method for various hydrophobic compounds. Further research into the casein micelle structure will not only provide a deeper understanding into its nature, but it may uncover new practical applications in the food industry and beyond.

Outline & Objectives

The casein micelle is a fundamental ingredient to the dairy industry. Therefore, it is of great importance to understand the structure of the casein micelle, and its behavior when subjected to different treatments and conditions. The second chapter of this dissertation deals with a three dimensional reconstruction of the native structure of the casein micelle as determined by cryo-transmission electron tomography.

It is also important to understand the interactions that the casein micelles have with other milk proteins. Chapter three of this dissertation presents the association of whey proteins with the casein micelles isolated from the raw skim milk of individual cows. This is a controversial new discovery, since it was widely accepted that whey proteins only interacted with the casein micelles as a result of heat or pH induced denaturation. The fourth chapter of the dissertation explores a method to facilitate the esterification of caseins from micelles in fluid milk. The esterification of casein has been shown to improve the stability of caseins at low pH values by shifting the isoelectric point of the caseins towards the alkaline. The fifth and final chapter of the dissertation outlines the effects of introducing a cold step in the fermentation of yogurt on the gel structure and whey holding capacity of the yogurt.

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CHAPTER II

CRYO-TRANSMISSION ELECTRON TOMOGRAPHY OF NATIVE CASEIN MICELLES FROM BOVINE MILK

This chapter is a lightly revised version of a paper by the same title submitted to the *Journal of Dairy Science* by Raymundo Trejo, Terje Dokland, Juan Luis Jurat-Fuentes and Federico M. Harte. The use of "our" in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the sample preparation, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the writing.

Abstract

Casein is the principal protein component in milk and an important ingredient in the food industry. In liquid milk, caseins are found as micelles of casein proteins and colloidal calcium nanoclusters. Casein micelles were isolated from raw skim milk by size exclusion chromatography and suspended in milk protein free serum produced via ultrafiltration (3 kDa MWCO) of raw skim milk. The micelles were imaged by cryo-electron microscopy and subjected to tomographic reconstruction methods to visualize the three-dimensional and internal organization of native casein micelles. This provided new insights into the internal architecture of the casein micelle that had not been apparent from prior cryo-transmission electron microscopy studies.This analysis demonstrated the presence of water filled cavities (*ca.* 20 to 30 nm diameter), channels (diameter larger than *ca.* 5 nm) and several hundred high density nanoclusters (6-12 nm diameter) within the interior of the micelles. No spherical protein submicellar structures were observed.

Introduction

Caseins are the primary protein components in milk. These proteins form large colloidal particles of 50-600 nm in diameter, known as casein micelles. Many of the technologically important properties of milk (e.g. its white color, stability to heat or ethanol and coagulation by rennet), are due to the properties of the casein micelles. Therefore, there is an economical and technological incentive in characterizing their properties and determining their structure (Fox and Brodkorb, 2008a). There is a general agreement that the main biological function of the casein micelle is to deliver calcium from mother to offspring, as milk is supersaturated with calcium found in the casein micelle as colloidal calcium phosphate (CCP).

Several models were proposed to describe the nano-structure of the casein micelle and recent detailed reviews can be found elsewhere (Dalgleish, 2011, Farrell et al., 2006a, Horne, 2009, Qi, 2007), including a discussion on the adequate use for the term “casein micelle” (Fox and Brodkorb, 2008b). The casein micelle has been described as containing submicelles linked together by CCP (Walstra, 1999) or as formed by CCP nanoclusters bound to phosphoserine-rich beta and alpha casein proteins (De Kruif CG, 2003) . The Horne model (Horne, 1998), considers the surface chemistry of the individual caseins and concludes that nonspecific protein-protein interactions also play a major role determining the casein micelle structure (Farrell et al., 2006b). In most cases, the nano-structure of the casein micelle was derived from X-ray or neutron scattering

studies (Hansen et al., 1996, Holt et al., 2003) or by transmission or scanning electron microscopy observations (Marchin et al., 2007, McMahon and Oommen, 2008, Qi, 2009). A major hurdle in obtaining a definite image of the native casein micelle through traditional electron microscopy techniques is the high water content of the micelles: on average 3.7 g water per gram of casein protein (Wong et al., 1996). Conventional electron microscopy requires preparation methods that involve sample dehydration, fixation, and staining, all of which potentially introduce artifacts that are difficult to discriminate or quantify. In cryogenic-transmission electron microscopy (Cryo-TEM), a thin layer of sample suspension is plunge-frozen in a suitable cryogenic fluid, resulting in the sample being embedded in a thin layer of vitreous ice, which retains the amorphous nature of the aqueous sample environment., followed by observation under cryogenic conditions, ensuring that the specimen is studied in a close to native state of preservation. Contrast in these specimens is generated not by the introduction of heavy atom stains, but through phase-contrast, which is produced from the coherent interference of the scattered and transmitted electron beams. Cryo-transmission electron tomography utilizes a series of Cryo-TEM images taken in angular increments to assemble a three-dimensional reconstruction of the object, including its internal features (Nudelman et al., 2011). The purpose of this study was to utilize cryo-transmission electron tomography to visualize the internal architecture of native casein micelles in three dimensions.

Materials and methods

Milk source & sample preparation

Raw milk samples were collected at a local dairy farm. Fat was removed by centrifugation at 4 °C (6,414X*g* for 20 min) and stored at 4 °C. Protein free serum (PFS) was prepared by tangential flow ultra-filtration (TFUF) of pasteurized skim milk using a 3KDa MWCO cellulose filter (PLBC Prep scale TFF Cartridge, Millipore, Billerica, MA). The PFS was preserved with 0.07% NaN_3 and stored at 4 °C.

Size exclusion chromatography

A size exclusion chromatography (SEC) column (Superdex 200 prep grade, GE Life Sciences, GE Health Care, Piscataway, USA) connected to a fast performance liquid chromatography (FPLC) unit (AKTA, GE Life Sciences, GE Health Care, Piscataway, USA) was used for the isolation of casein micelles. Raw skim milk samples were loaded into the column and iced-cold PFS (prepared as previously mentioned) was used for elution to preserve the native conditions. Separation was achieved using a flow rate of 1.0 ml/min for 2 column volumes (240 ml total), collecting 2 ml fractions. The protein elution chromatograph was determined using absorbance set at 280nm. Collected fractions containing the casein micelles (confirmed by SDS-PAGE) were stored at 4 °C.

Cryo-transmission electron tomography

Immediately before imaging by cryo-TEM, a sample was prepared

containing 4 µl of casein micelle isolates (from FPLC), 18 µl of protein free serum (permeate from 3.5 KDa TFUF of skim milk), 36 µl distilled water, 2 µl of 6 nm BSA-gold tracer (Electron Microscopy Sciences, Hatfield, PA), and 2 µl of 15 nm ProteinA-gold (Electron Microscopy Sciences, Hatfield, PA). The gold particles were added as fiducial markers for the tomographic reconstruction. 3 µl of this suspension was pipetted onto 200 mesh holey grids (R2/1 Quantifoil Micro Tools, Jena, Germany), blotted briefly, frozen by plunging into liquid ethane at –182°C, transferred to a Gatan 626 cryo-sample holder, and observed in an FEI Tecnai F20 electron microscope operated at 200kV (Dokland and Ng, 2006). Tomographic reconstruction was carried out as previously described (Spilman et al., 2009). Tilt series were acquired semi-automatically using the FEI Xplore3D software at a magnification of 38,000× and nominal defocus of 6 µm and collected over an angular range of up to 138° in 2° steps. The total electron dose for the entire tilt series was 80–120 $e^{-}/Å^{2}$, or about 1–2 $e^{-}/Å^{2}$ for each image (McEwen et al., 1995). Images were recorded on a Gatan Ultrascan 4k x 4k CCD camera operated in binned mode, resulting in a pixel size of 7.9 Å on the sample. Tomographic data processing was done with the IMOD software package (IMOD 4.1.8, Boulder, CO) (Kremer et al., 1996). No phase correction was used, since all the data used was within the first zero of the CTF, but a 22 Å low pass filter was applied during reconstruction. The casein micelle reconstructions were visualized using the UCSF Chimera visualization and analysis software package (Goddard et al., 2007; Huang, 1996). The three dimensional reconstruction was visualized at two different density cutoff levels to differentiate between the protein and the

colloidal nanoclusters. At 1.76 standard deviations above the mean, only the high density colloidal nanoclusters were apparent. A Gaussian smoothing filter was applied and the map was visualized at 0.61 standard deviations above the mean to show the protein nanoclusters. The size of the calcium nanoclusters and the diameter of the internal structures of the casein micelle were measured utilizing Image J image analysis software package (ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). The size and number of calcium nanoclusters is an average obtained from examining several casein micelles. The average diameter of the internal structures was calculated in the same manner. Video files showing unaligned and aligned TEM micrographs, tomogram of casein micelles and casein micelle reconstruction can be found in http://web.utk.edu/~fede/casein-micelle.html

Results and discussion

A typical cryo-TEM image of the native casein micelles at 0^{o} tilt is shown in **Figure 2.1**. The three dimensional tomograms was assembled from a series of such images at tilt angles from –64° to +64°. A central section through one of the tomograms is shown in **Figure 2.2.** The cryo-transmission electron tomogram of the casein micelle shows that the micelle is highly porous throughout its structure, as previously reported (McMahon and Oommen, 2008). The average thickness of the casein micelles in the tomograms is 100 nm. The internal structures within the casein micelle exhibited a random arrangement resulting in the presence of irregular water-filled channels larger than *ca.* 5 nm (**Figure 2.3A**

and 2.3B) and inner cavities of *ca*. 20 to 30 nm in diameter **(Figure 2.3A and 2.3C**). The existence of these structures has been previously suggested by Dalgleish (2011), who postulated that the hydrophobic interactions of the proteins would lead to an uneven distribution of water within the interior of the micelle and the formation of water channels. The existence of cavities within the micelle was also proposed by McMahon and Oommen (2008) as part of the interlocking lattice model of the casein micelle structure.

Isosurface representations of the three-dimensional reconstruction are shown in **Figure 2.4**. The cylindrical appearance of the micelle is due to the deformation caused by the surface pressure of the 50-200 nm thick embedding film of vitreous ice resulting in the spherical micelles being compressed into a more cylindrical shape. Nano-rheological studies of casein micelles using vibrating atomic force microscopy yielded Young's modulus of 0.06 to 0.08 MPa, typical of easily deformable polymeric soft foams (Helstad et al., 2005). This compressive deformation occurred parallel to the observation direction and has been reported in previous cryo-TEM studies of casein micelles (Marchin et al., 2007).

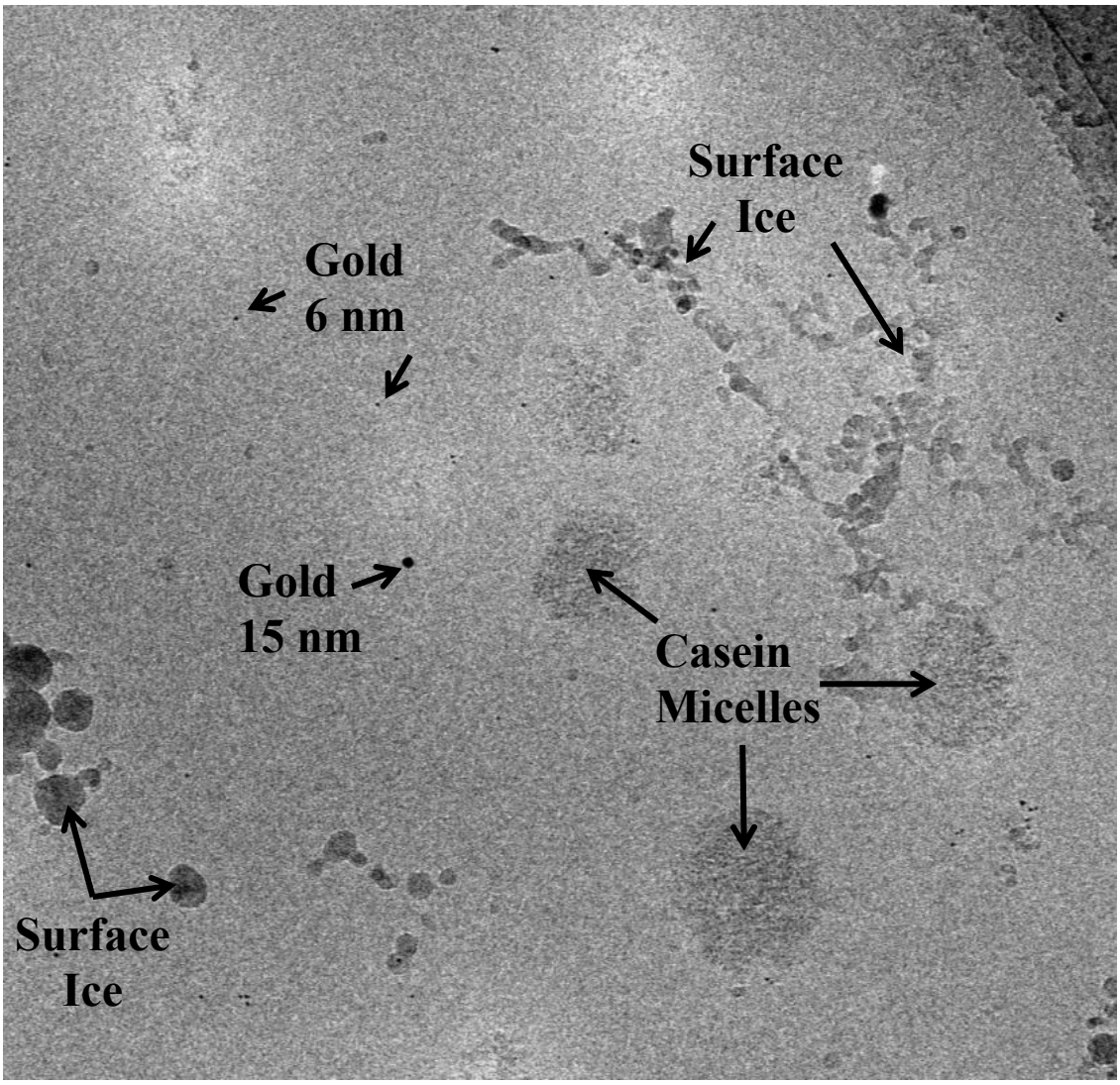

Figure 2.1. *Cryo-transmission electron tomography image of native casein micelles. Three casein micelles, 6- and 15-nm gold particles, and ice contaminants are indicated*

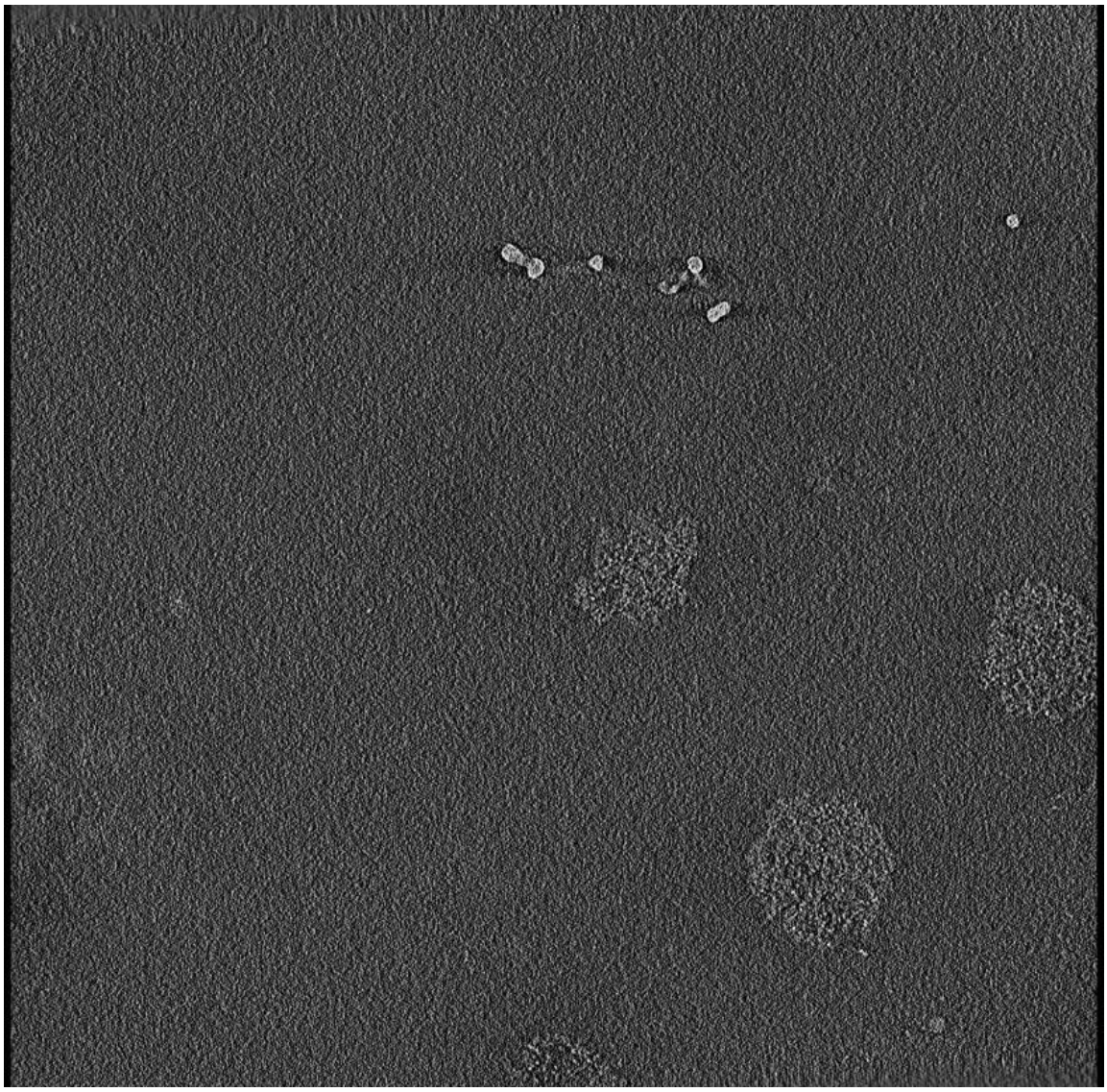

Figure 2.2. *A 4.74 nm thick slice through the center of one cryo-transmission electron tomogram, showing three casein micelles. The contrast of the figure is inverted relative to the micrograph shown in Figure 1.*

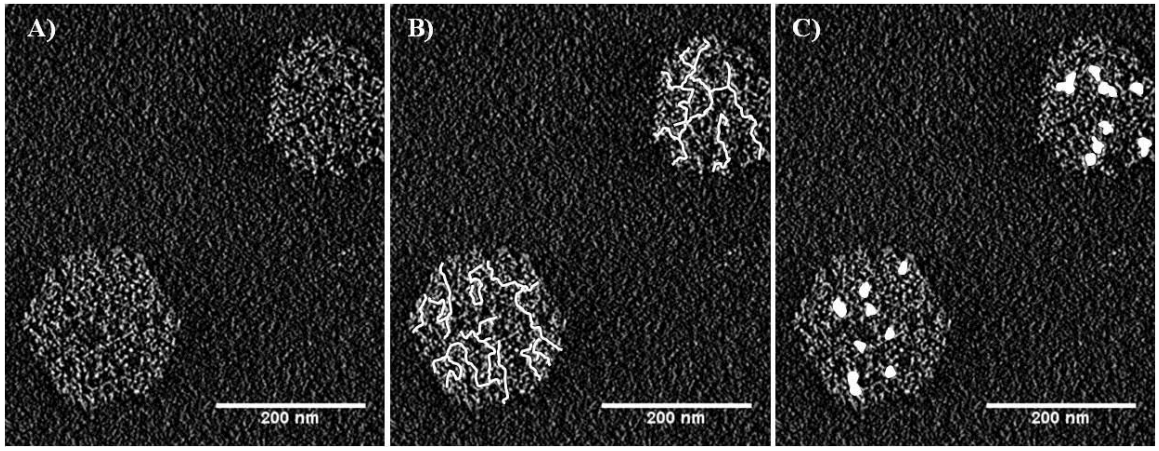

Figure 2.3. A) *4.74 nm slabs through the centers of two micelles from the cryo-transmission electron tomograms,* ***B)*** *A highlighting of the internal channels in the native casein micelles in the same image,* ***C)*** *A highlighting of the internal cavities in the native casein micelles in the same image. Scale bar 200 nm*

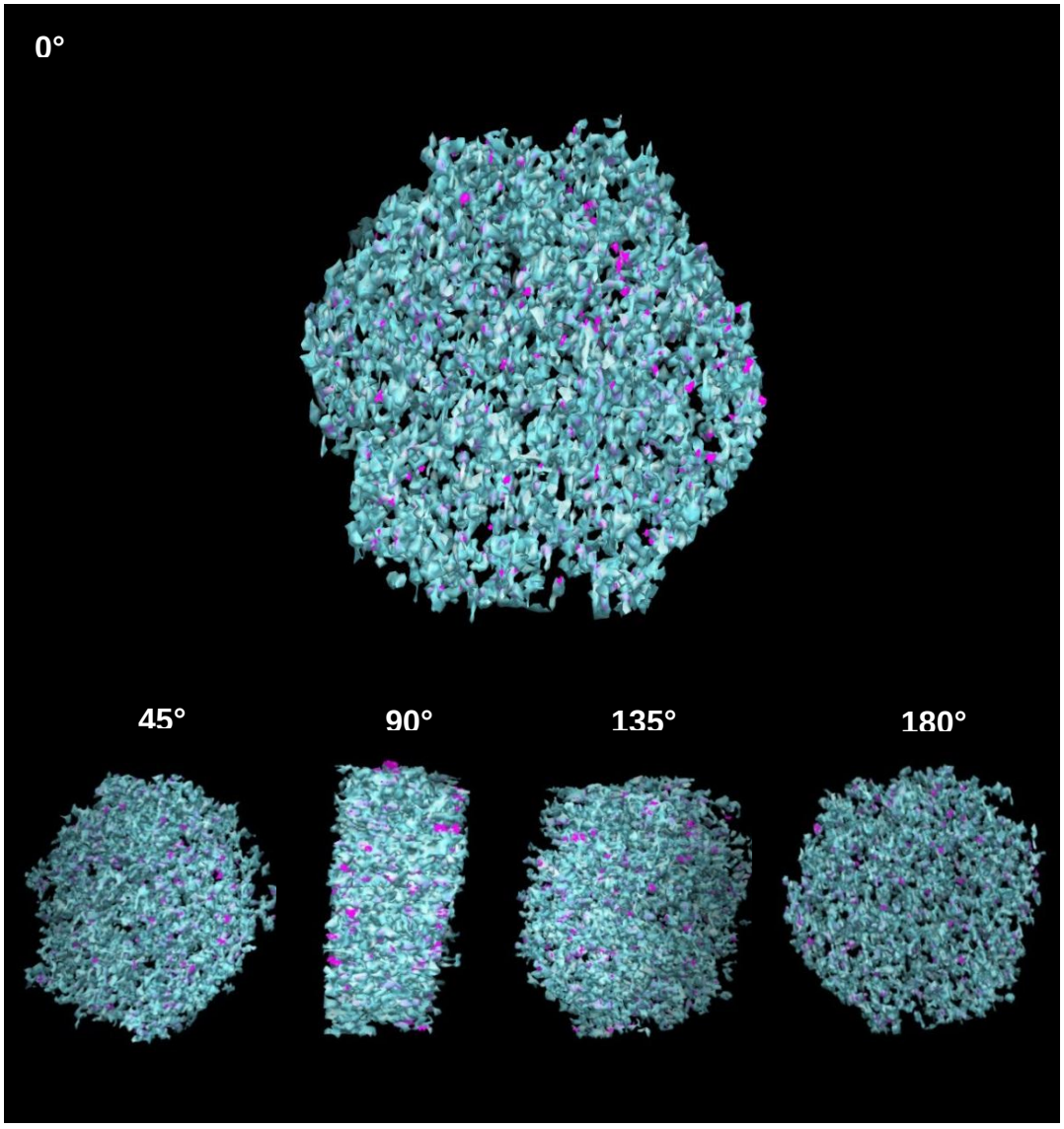

Figure 2.4. *Isosurface representation of the tomogram of the native casein micelle, shown at five different angles. The blue surface is shown at a cutoff level of 0.61 standard deviations above the mean, while the pink surface is at 1.76 standard deviations above the mean, highlighting the positions of the higher density colloidal calcium phosphate. The transparency of the blue surfaces has been decreased to 0.45 to facilitate the viewing of the colloidal calcium phosphate*

Colloidal calcium phosphate clusters (represented as pink particles in **Figure 2.4**) were found evenly distributed throughout the micelle with number ranging from 700 to 800, as suggested by De Kruif and Holt (2003). The diameter of the colloidal calcium nanoclusters ranged from *ca.* 6 to 12 nm, larger than the 4.8 nm diameter reported by Mahon and Oommen (2008) but similar to the calcium phosphate aggregates observed by Houjo et al. (1977). An isosurface representation of a 35 nm thick slab through the casein micelle reconstruction (**Figure 2.5**) shows the extensive number of water filled channels and cavities within the inner structure of the native casein micelle.

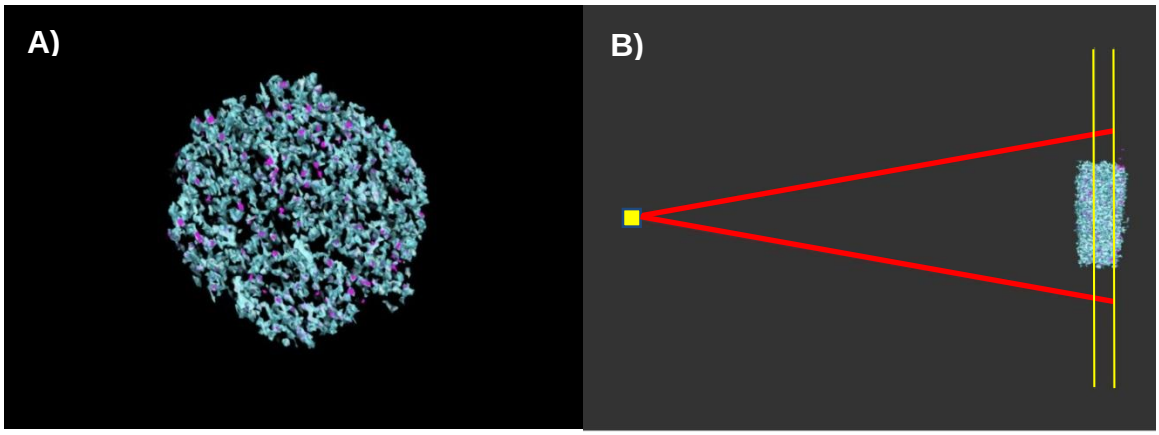

Figure 2.5. ***A)*** *Isosurface representation of a 35 nm slab through the native casein micelle tomogram, showing the channels and cavities (black areas) found within the micelle.* ***B)*** *Markers indicating the location of the slab within the interior of the casein micelle.*

Conclusion

In this study, cryo-transmission electron tomographic reconstruction has allowed us to examine the interior of the native casein micelle. The tomograms demonstrated the presence of water filled cavities and channels within the interior of the native micelle structure, and the absence of any spherical protein sub-structures. These observations further question the existence of sub-micelles within the casein micelle, and confirm the existence of channels and inner cavities. These inner cavities, which are interconnected by wide diameter channels, may explain the dynamic nature of the native casein micelles and its ability to release and retain β-casein and other macromolecules (Sahu et al., 2008)

Acknowledgements

3D Casein Micelle images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081). This research was partially supported by the University of Tennessee Institute Of Agriculture and by the Dairy Research Institute.

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CHAPTER III

INTERACTION OF CASEIN MICELLES FROM RAW SKIM MILK WITH WHEY PROTEINS AND THE HYDROPHOBIC PEPTIDE VALINOMYCIN

This chapter is a lightly revised version of a paper by the same title to be submitted to the *Journal of Dairy Science* by Raymundo Trejo, Maneesha Mohan Sasha Wilkinson, Juan Luis Jurat-Fuentes, and Federico M. Harte. The use of "our" in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the experimental work, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the writing.

Abstract

The principal protein in bovine milk is casein. In liquid milk, it is found in the form of micelles. Casein is an important ingredient to the dairy industry; however, the actual structure of the casein micelle has not been definitely established. One of the structural models proposed for the casein micelle consists of a highly porous structure of the micelle, and the existence of channels and cavities in the casein micelle was confirmed via cryo-electron transmission tomography. Given the diameter of these channels and cavities, it would be possible for whey proteins or peptides to interact with the micelle. To investigate this interaction, casein micelles from the raw skim milk of three individual cows, at native pH (6.8) and pH 5.0, were isolated via size exclusion chromatography (SEC). Analysis of the SEC elution peaks by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under both reducing and non-reducing conditions established the protein components of the elution peaks. Whey proteins were found associated with the casein micelles in the elution fractions at both pH values under reducing conditions. However, under non-reducing conditions, the bands for the whey proteins were only seen at pH 6.8 and were not visible at pH 5.0. This indicates that the nature of the whey protein-micelle association is mainly via hydrophobic interactions at native pH and via disulfide bonds at the lower pH.

To further evaluate the casein micelle interactions, raw skim milk was combined with the hydrophobic peptide valinomycin (MW= 1111.32; logP= 5.92), and the

micelles were isolated by microfiltration with a 0.22 μm tangential flow filter. SDS-PAGE was utilized to confirm the presence of valinomycin with the isolated micelles. However, no bands for valinomycin were found in the gels. This could be due to the peptide not associating with the micelle; however, it could also be due to limitations of the SDS-PAGE to properly identify the bands.

The presence of whey proteins in association with native casein micelles is a significant finding since it stands contrary to the previously thought composition of the casein micelles. A possible avenue for the association is the highly porous structure of the micelle. However, it remains unclear if it is possible to exploit the porosity and binding properties of the micelle for associating a hydrophobic peptide with the native micelle.

Introduction

Casein, the principal protein in bovine milk, is found in liquid milk in the form of micelles. These casein micelles are loose, highly hydrated aggregation of caseins with an average size of 200-300 nm (Fox and Brodkorb, 2008). Several models of the casein micelle structure have been proposed (DeKruif, 2003, Horne, 1998, Walstra, 1999). Based on observations from cryo-electron transmission microscopy, McMahon et al. proposed an open lattice model with a highly porous structure, (McMahon and Oommen, 2008). The presence of channels and cavities within the casein micelle was originally proposed by Dalgleish, based on the hydrophobic forces that occur within the casein micelle (Dalgleish, 2011). As previously shown, the existence of these channels and cavities was confirmed via cryo-electron transmission tomography (Trejo et al., 2011). Given the 5-10 nm average diameter of the channels and 20-30nm average diameter of the cavities, it would be possible for the whey proteins to transit in and out of the casein micelle. Previously, Dalgleish et.al., studied the association of whey proteins with the casein micelles by analyzing isolated casein micelles by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). These authors mentioned the presence of small amounts of whey proteins associated with the micelles. However, since the source material was skim milk powder, they attributed the presence of these proteins to the heat treatment utilized to produce the milk powder (Dalgleish et al., 2004). Our attempts to isolate casein micelles by ultra-filtration and microfiltration always encountered contamination by whey proteins independently of the pore

size of the filter being used. The whey proteins were also found in commercial native casein isolates.

Boulet et. al., (1970) demonstrated the use of size exclusion chromatography for the isolation of casein micelles. In this method the casein micelles were detected in the first elution peak obtained from size exclusion chromatography separation of a skim milk sample (Boulet et al., 1970) . This was later verified by other researchers utilizing modifications of the Boulet method (Britten et al., 1986, Gupta, 1983). Furthermore, SEC has also been used successfully in the past to separate whey proteins (Hill et al., 1986).

The interactions between casein micelles and whey proteins in milk have been reported in the past (Smits and Vanbrouwershaven, 1980). Under heating conditions (~>75^{o}C), β-lactoglobulin denatures and forms disulphide bonds with the κ-casein layer of the casein micelle. Several authors suggested that such interaction was only due to effects of heat treatment, and that pH had an effect on the rate of attachment (Anema and Li, 2003, Corredig and Dalgleish, 1996, Oldfield et al., 2000). It has been demonstrated that in acidified conditions (pH 5.6 - 5.2) and cold temperatures (≤5 ^{o}C) β-casein migrates out of the micelle due to a weakening of the hydrophobic interactions in the micelle (Dalgleish and Law, 1988). Also, when the pH of milk is reduced to 5.6 – 5.2, the colloidal calcium migrates out of the micelle (Dalgleish and Law, 1989). If the whey proteins are associated with the casein micelle, then it would be possible that a lowering of the pH and temperature would result in a behavior similar to that of β-casein. This would lead to a decrease in the number of associated whey proteins.

The effectiveness of the casein micelles from processed milk in binding other compounds has been previously reported (Livney, 2010). Given the presence of the channels and cavities in the micelle, it would be possible for a hydrophobic peptide to move into the micelle due to the higher concentration of hydrophobic zones found within the micelle. The purpose of this study was to test whether a native interaction between the casein micelles and the whey proteins exist, the effect that a reduced pH could have on that interaction, and the possibility that a small hydrophobic peptide could migrate into the casein micelle.

Materials & Methods

Milk source & sample preparation

Raw milk samples from three individual cows of the same age and stage of lactation were collected from the University of Tennessee research dairy farm. All milk samples were collected directly downstream from the milking apparatus without any of the milk mixing with milk from other cows. The milk was stored in ice for transport immediately after collection, and kept refrigerated at 4 °C until further processing. All experiments were conducted with milk from the same three individual cows. Fat was removed from the milk samples by centrifugation at 4 °C (6414X*g* for 20 min) and stored at 4 °C. The native pH of the raw skim milk sample was measured and recorded to be 6.8. Prior to testing, two aliquots of the milk samples were separated and, to evaluate the effects of pH on the micelle-whey proteins interaction, the pH of one of the milk samples was adjusted to 5.0; while the other was kept at the native pH. The pH of the samples

was adjusted under constant agitation using a 1.0 N HCl solution (Fisher Scientific, Fair Lawn, NJ). Protein free serum (PFS) was prepared from commercially available pasteurized skim milk obtained from a local grocery store and kept at 4 °C until processing. The PFS was obtained by tangential flow ultra-filtration using a 3KDa MWCO cellulose filter (PLBC Prep scale TFF Cartridge, Millipore, Billerica, MA). The PFS was stored at 4 °C. Absence of proteins in PFS was confirmed using 12% acrylamide SDS-PAGE with silver staining. Prior to testing, the pH of the required volume of PFS was adjusted to match the pH of the milk sample being tested. As with the raw skim milk samples, the pH was adjusted using a 1.0 N HCl solution.

Size exclusion chromatography

A Superdex 200 prep grade SEC column (GE Healthcare Life Sciences, Piscataway, NJ) connected to an AKTA (GE Healthcare Life Sciences, Piscataway, NJ) fast performance liquid chromatography (FPLC) unit was used for size exclusion purification of milk samples. Raw skim milk samples of the desired pH were loaded into the FPLC and PFS of the same pH as the milk sample was used for elution. Separation was achieved using a flow rate of 1.0 ml/min for 2 column volumes (400 ml total), collecting 2 ml fractions and monitoring absorbance at 280 nm to monitor protein elution. Collected fractions were stored at 4 °C until analyzed. All samples were analyzed within 24 hours of fractionation. Two replicate runs were performed for each of the samples.

Microfiltration & Hydrophobic Peptide Interaction

In order to evaluate if a small hydrophobic peptide could enter the casein micelle via the channels and cavities, a low molecular weight hydrophobic peptide, valinomycin (MW= 1111.32; logP= 5.92; Sigma Aldrich, St. Louis, MO), was selected due to its molecular weight and hydrophobicity. Valinomycin is non-soluble in water. Therefore, it was dissolved via vortexing in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml prior to mixing with the raw skim milk sample. Two milliliters of the valinomycin/DMSO solution werethen added to a 10 ml aliquot of raw skim milk and vortexed for 2 minutes. To promote the interaction between the peptide and the micelles, the resulting 12 ml peptide-milk mixture was then mixed utilizing a Polytron® PT 10-35 GT rotary homogenizer (5000 rpm; Kinematica, Lucern, Switzerland) for 5 minutes. Then, one 12 ml peptide-milk sample was diluted with 38 ml of a buffer (buffer A) containing 20 mM imidazole and 10 mM calcium chloride (pH 6.8) to a final volume of 50 ml. The second milk sample was diluted with38 ml of a buffer (buffer B) containing 20 mM imidazole and 10 mM calcium chloride and 5% (v/v) of DMSO (pH 6.8) to a final volume of 50 ml. Both of the samples were kept overnight at 4 ^{o}C prior to filtration. Another identical pair of samples was prepared just prior to filtration. To isolate the micelles via microfiltration, the samples were filtered utilizing a 0.22 μm polivinylidene fluoride filter (Pellicon® XL Millipore, Billerica, MA). The permeate was discarded, while the retentate was recirculated and the lost volume replaced with the appropriate buffer. After the full volume of the sample was replaced 4 times, the samples were removed,

frozen at -40 °C, and lyophilized to remove the water and DMSO. The samples were then re-suspended in 5 ml of distilled deionized water prior to gel electrophoresis. All experiments were done in triplicate.

SDS-PAGE

The SEC fractions corresponding to the elution peaks observed in the chromatograph were combined and prepared for electrophoresis as follows. For SDS-PAGE under denaturing and reducing conditions, a sample loading buffer was prepared using 0.5 M Tris-HCL pH 6.8, glycerol, 10% (w/v) SDS, β-mercaptoethanol, and 0.5% (w/v) bromophenol blue in water (Fisher Scientific, Fair Lawn, NJ). The samples were heat denatured for 5 minutes at 95 °C prior to electrophoresis in 15% acrylamide SDS-PAGE ready gels (Biorad, Hercules, CA). Sample volumes of 15 µl were loaded into each well and electrophoresis run at constant 200 volts for 38 minutes. For native non-reducing conditions, the loading buffer for these samples was prepared as above except that no β-mercaptoethanol was used in the loading buffer and its volume was replaced with DI water. Also, the samples were not heat denatured prior to gel electrophoresis. Gels were stained using manufacturer's protocol (Silver stain plus, Biorad, Hercules, CA). Protein concentration in each sample was estimated using densitometry (ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/) with standard samples containing 0.1, 0.25, 0.50, 0.75, and 1.0 mg/ml of β-Casein, β-lactoglobulin, and α-lactalbumin (Sigma Aldrich, St. Louis, MO). All experiments were performed in triplicate. The identity of the

proteins was confirmed via commercial protein isolates for β-casein, β-lactoglobulin, and α-lactalbumin (Sigma Aldrich, St. Louis, MO).

For peptide SDS-PAGE sample preparation, 250 µl of the samples were mixed with 250 µl of a loading buffer containing 200 mM Tris-HCl, pH 6.8, 2% SDS, 40% glycerol, 0.04% Coomassie Brilliant Blue G-250, and 2% β-mercaptoethanol. The samples were heat denatured for 5 minutes at 95 °C prior to electrophoresis. 10-20% TRIS-Tricine ready gels were used for the electrophoresis (12 well precast gel, Biorad, Hercules, CA). Sample volumes of 18 µl were loaded into wells and electrophoresis was run at 100 volts for 100 minutes. After electrophoresis, the gels were fixed in a buffer containing 40% methanol and 10% acetic acid (v/v). Following the fixing step, the gels were stained for 60 minutes in a solution containing 0.025% (w/v) Coomassie blue G-250 (Biorad, Hercules, CA) and 10% (v/v) acetic acid. The gels were then de-stained with a 10% (v/v) acetic acid solution until the peptide bands became visible. All experiments were done in triplicate.

Results and discussion

Size exclusion chromatography

The different protein components of milk were successfully isolated via SEC. **Figure 3.1** shows a representative chromatograph illustrating the proteins found in the fractions when the original sample was kept at the native pH of milk (6.8). The much larger casein micelles eluted first and were found in the first peak, which also contained whey proteins. This suggested the possibility that the whey

proteins were associated with the casein micelles, although in very small amounts. The second SEC peak contained bovine serum albumin (BSA) and β-casein. The presence of the β-casein was expected due to the temperatures at which the samples were stored prior to the SEC (Dalgleish and Law, 1988). No β-lactoglobulin or α-lactalbumin was found in these fractions. The fractions containing the third elution peak contained primarily β-lactoglobulin and a small amount of α-lactalbumin. This was reversed for the fractions containing the fourth elution peak, which contained a large amount of α-lactalbumin and a small amount of β-lactoglobulin. No caseins were found in the third or fourth elution peaks. As seen in **Figure 3.2**, the results were consistent for all of the cows. The results were also consistent for all of the replicates.

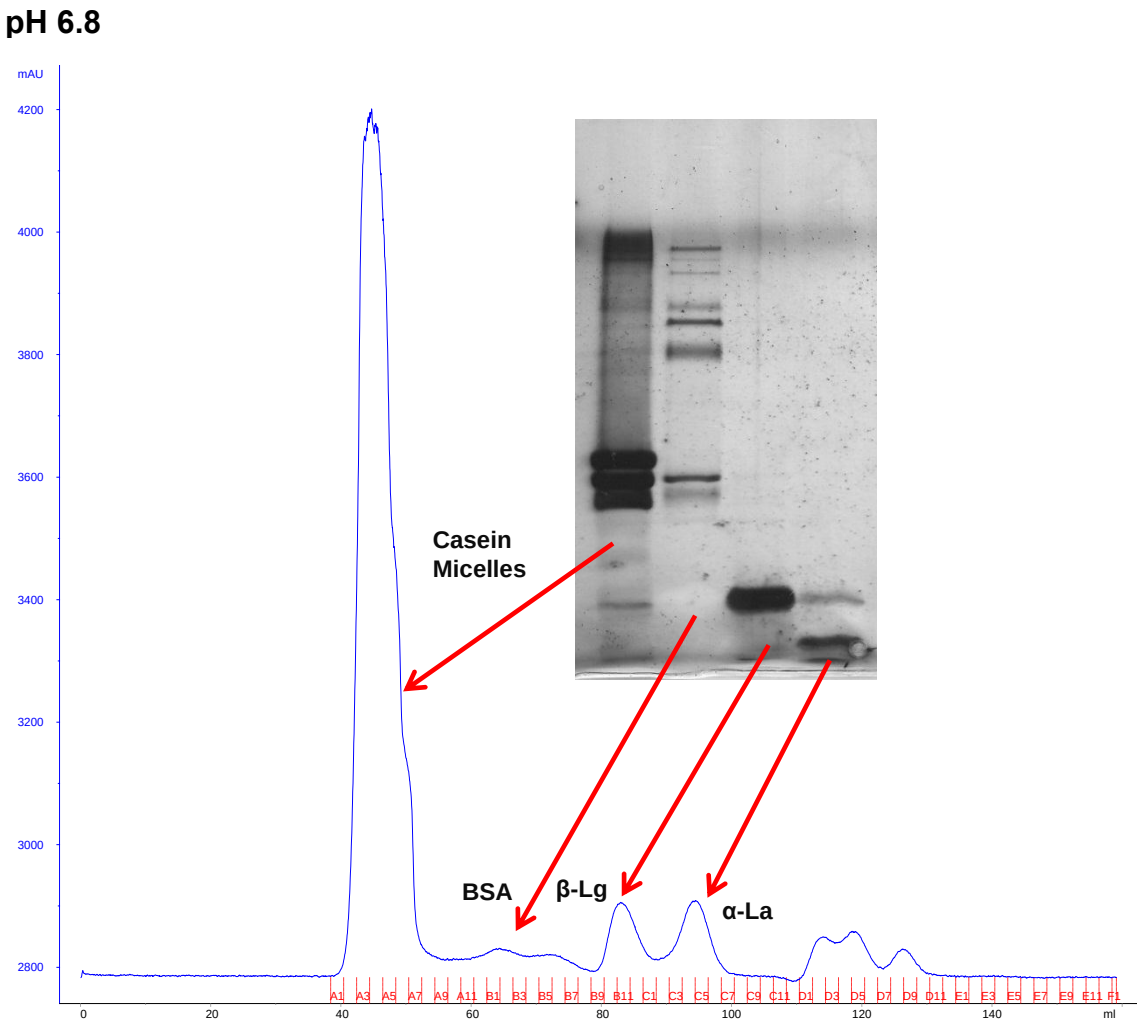

Figure 3.1. *Representative SDS-15% Polyacrylamide gel electrophoresis under reducing & denaturing conditions of the fractions containing the elution peaks detected (A=280nm) from a size exclusion chromatography elution of a raw skim sample from a Holstein cow (Cow 1) at native pH 6.8 value. The identity of the individual proteins was confirmed via isolated standards.*

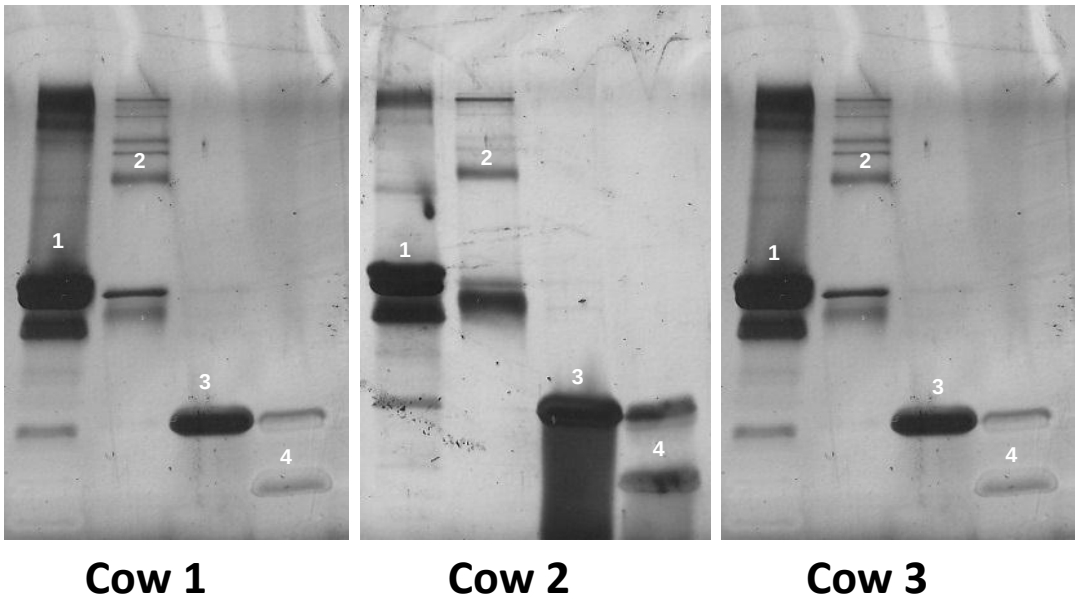

Figure 3.2. *SDS-15% Polyacrylamide gel electrophoresis under reducing & denaturing conditions of the protein components of the size exclusion chromatography elution peaks from the raw skim milk of three individual cows (pH 6.8). 1 = Casein, 2 = Bovine Serum Albumin, 3 = β-lactoglobulin, 4 = α-lactalbumin*

Lowering the pH of the samples altered the protein pattern detected in the elution peaks. **Figure 3.3** shows a representative chromatograph and the proteins found in the fractions containing the peaks in the elution chromatographs when the samples were adjusted to pH 5.0. As seen in **Figure 3.4**, the results were consistent for all of the cows. The results were also consistent for all of the replicates.

As observed for the pH 6.8 samples, at pH 5.0, the casein micelles eluted first and whey proteins where found associated with the isolated casein micelles. The fractions containing the second elution peak contained BSA and β-casein; however, β-lactoglobulin proteins were also found in these fractions. The third and fourth elution peaks had the same proteins as the ones detected for the samples at native pH. The presence of β-lactoglobulin in the second peak may be explained by the proteins moving out of the casein micelle. However, it is known that at pH 5.0- 5.1 β-lactoglobulin has the tendency to self-associate into octamers while at neutral pH it is found in dimers (Cheison et al., 2011, McKenzie and Sawyer, 1967, Taulier and Chalikian, 2001). This increase of size of the octamers versus the dimers would result in β-Lactoglobulin being found alongside the larger β-casein and BSA proteins.

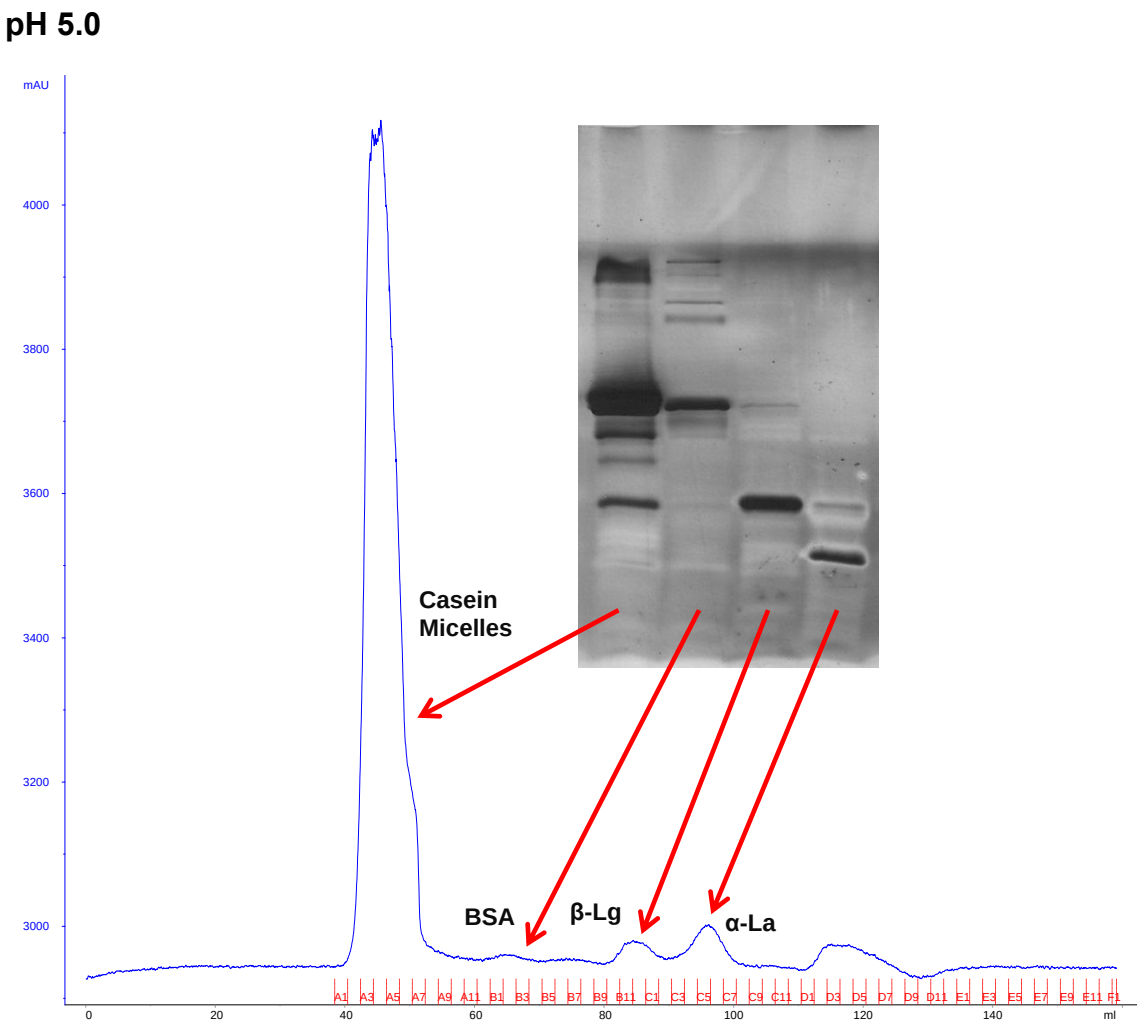

Figure 3.3. *Representative SDS-15% Polyacrylamide gel electrophoresis under reducing & denaturing conditions of the fractions containing the elution peaks detected (A=280nm) from a size exclusion chromatography elution of a raw skim sample from a Holstein cow (Cow 1) at an adjusted pH value of 5.0. The identity of the individual proteins was confirmed via isolated standards.*

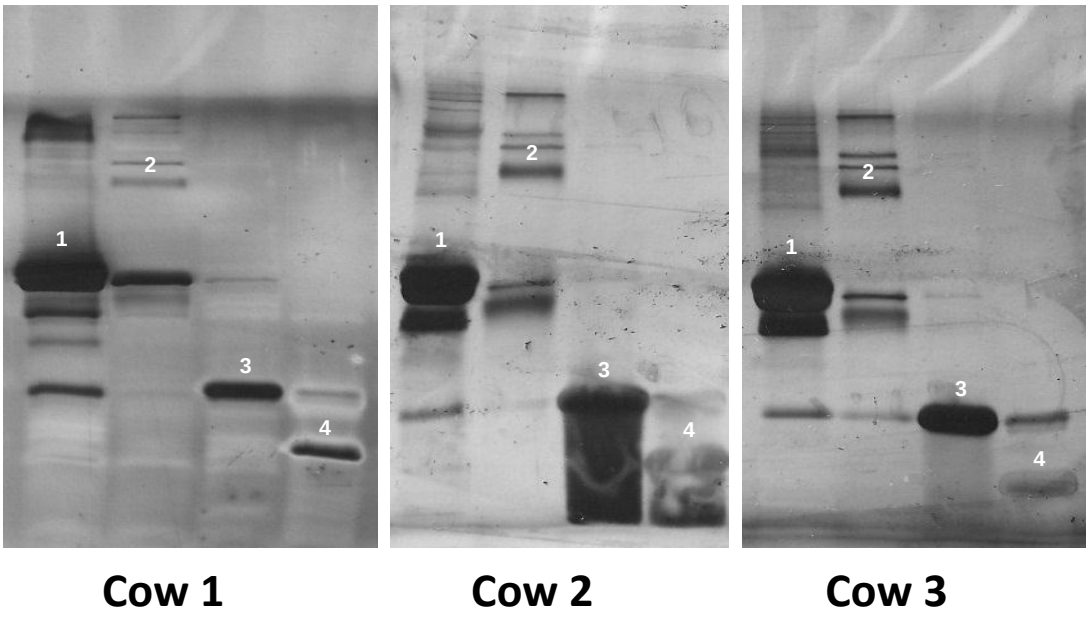

Figure 3.4*. SDS-15% Polyacrylamide gel electrophoresis under reducing & denaturing conditions of the protein components of the size exclusion chromatography elution peaks from the raw skim milk of three individual cows (pH 5.0). 1 = Casein, 2 = Bovine Serum Albumin, 3 = β-lactoglobulin, 4 = α-lactalbumin*

F**igure 3.5** shows the relative intensity of the bands for β-casein, β-lactoglobulin, and α-lactalbumin isolated via SDS-15 % Polyacrylamide gel electrophoresis under reducing and denaturing conditions of the SEC fractions containing the isolated casein micelles for the individual cows tested at pH 6.8 and 5.0**.** The variation among the individual cows was too large to observe any major change in the amount of whey proteins associated with the isolated casein micelles at either of the pH values tested. In our original hypothesis, the whey proteins would exhibit a similar behavior to β-casein, which migrates out of the casein micelle when at pH values of 5.6 – 5.2 and low temperatures (Dalgleish and Law, 1989). However, the relative intensity of the fractions shows that the ratio of β-casein to whey proteins found in the casein micelle fraction remains fairly constant regardless of the pH value of the samples.

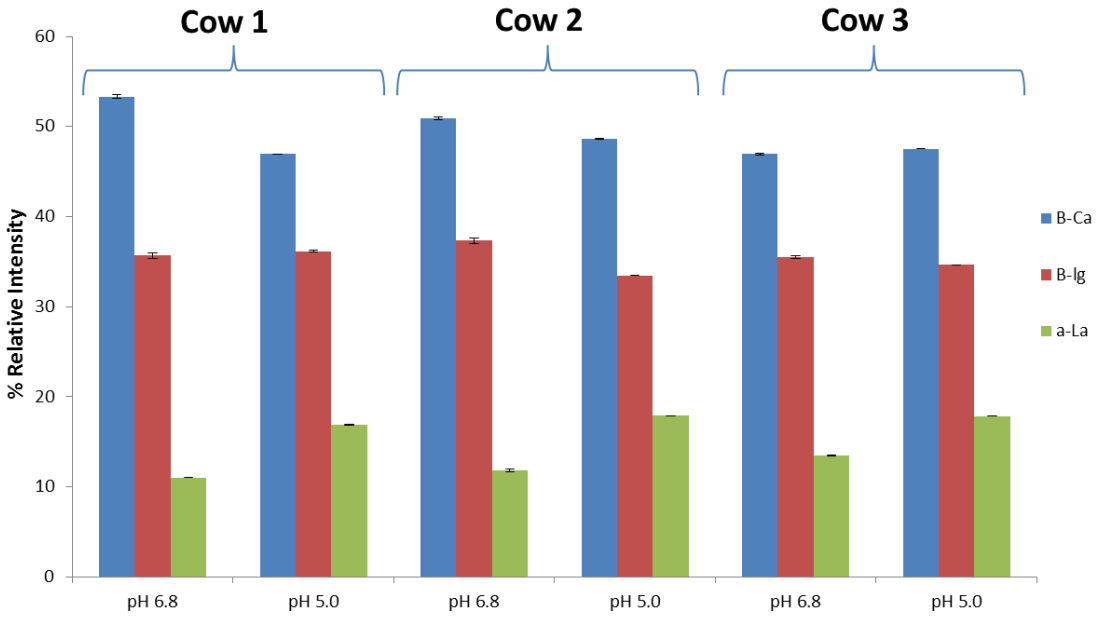

Figure 3.5. Relative intensity of the bands for β-casein, β-lactoglobulin, and α-lactalbumin isolated via SDS-15 % Polyacrylamide gel electrophoresis under reducing and denaturing conditions of the size exclusion chromatography fractions containing the isolated casein micelle for the individual cows tested at pH 6.8 and 5.0.

In order to further evaluate the nature of the whey protein-micelle interaction, the fractions containing the SEC isolated casein micelles were analysed via SDS-PAGE under reducing/denaturing and non-reducing/non-denaturing conditions. **Figure 3.6A** presents SDS-15 % PAGE under reducing/denaturing conditions for the fractions in the first elution peak from milk at both pH values tested, which contains the casein micelles. The whey proteins were found in all fractions at both pH values. **Figure 3.6B** presents the SDS-15% PAGE of the same samples run under non-reducing/non-denaturing conditions. Whey proteins were present in the samples isolated at native pH (6.8), but not in the samples isolated at pH 5.0. This indicated that the interaction between the caseins and the whey proteins at native pH is most likely due to hydrophobic interactions, which are disrupted by the presence of SDS in the loading buffer. This allows for the bands to separate from the caseins. At the lower pH value, the whey proteins did not separate from the caseins. This indicates that at this pH value the whey proteins interact with the caseins via disulfide bonds since reducing conditions were required for the bands to be visible. Furthermore, bands for proteins with large molecular weights (> 66 KDa) are visible in the gels run under non-reducing/non-denaturing conditions that are not visible in the gels ran under reducing/denaturing conditions. These bands could contain the whey protein-casein conjugates; however, with it is not possible to determine the identity of the proteins with the methods employed in this study. A possible method for protein identification would be to utilize a whey protein specific antibody.

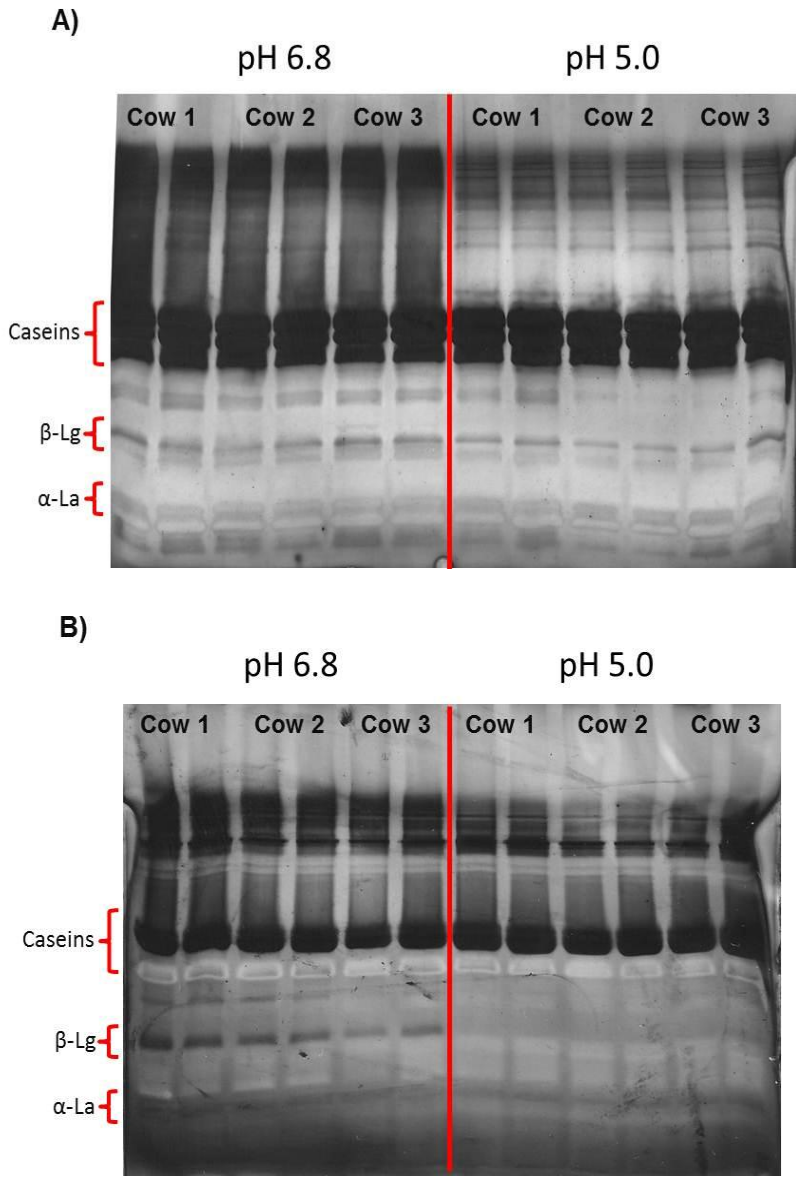

Figure 3.6. A) *SDS-15% Polyacrylamide gel electrophoresis under reducing/denaturing conditions for the fractions from the first elution peak obtained from milk samples at pH 6.8 and 5.0. B**)** SDS-15% Polyacrylamide gel electrophoresis under non-reducing/non-denaturing conditions for the fractions from the first elution peak obtained from milk samples at pH 6.8 and 5.0.*

Given the size of the channels in the casein micelle, it would be possible for a small hydrophobic peptide to migrate towards the more hydrophobic environment found within the micelle. Valinomycin, with its small molecular weight and hydrophobic properties would be great candidate the verify if this behavior was possible. The binding abilities of the casein micelle are very well known, and they are regularly utilized in order to employ the micelles as delivery methods for bioactive compounds, vitamins, and flavors, such as curcumin (Livney, 2010, Sahu et al., 2008, Semo et al., 2007). Therefore, we expected the hydrophobic peptide valinomycin to complex with the casein micelles. However, as seen in **Figure 3.7.,** we did not detect evidence of the presence of valinomycin associated with the casein micelles with the SDS-PAGE analysis we utilized. One potential explanation for this observation is that the particle size of the valinomycin dissolved in DMSO was too large to enter the casein micelle, but not large enough to avoid being filtered out during the microfiltration step. Another possible explanation for the lack of visible valinomycin bands in the gel could be weak binding of the coomasie stain to the valinomycin. Coomasie blue has a strong binding ability to amino acids, but it binds more strongly to the basic amino acids Arginine, Lysine, and Histidine (Diezel et al., 1972). Valinomycin is composed entirely of the amino acid Valine, which coomasie blue would not bind as effectively as it does to other proteins that contain a more diverse amino acid sequence. Given the small amounts of valinomycin that would be present in the gel and the weak binding of the stain, the faint bands of the valinomycin would be lost during the de-staining process. In order to conclusively establish or discount

the association of the peptide with the casein micelle, other analytic methods, such as high pressure liquid chromatography, would need to be utilized. Unfortunately, that was beyond the scope of this project.

Although valinomycin was not found in the casein micelles, the whey proteins were found in all of the samples. By employing a different isolation method, it is shown that the whey proteins are found associated with the casein micelles regardless of the isolation method. By utilizing raw skim milk in the isolation of the casein micelles via microfiltration, it further demonstrates that the casein micelles and the whey proteins associate in the cow during the production of milk.

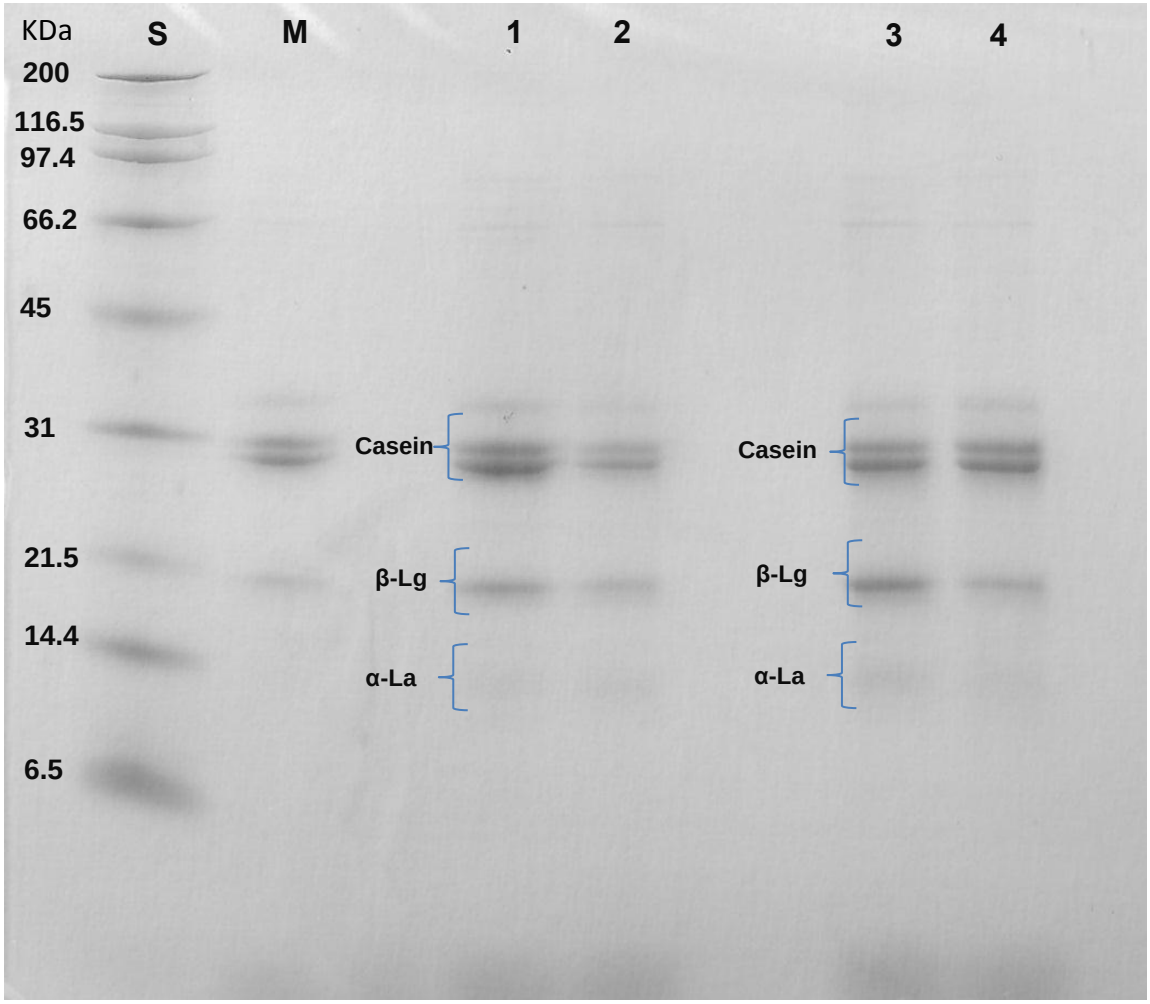

Figure 3.7. Peptide 10-20% Tris-Tricine SDS-Polyacrylamide gel electrophoresis of casein micelles isolated via microfiltration (0.22 µm tangential flow filter) from a valinomycin-raw skim milk mixture. S= Molecular size standard, M= Raw skim milk, 1 = sample stored overnight in a 20 mM imidazole and 10 mM calcium chloride buffer (pH 6.8), 2 = sample stored overnight in a 20 mM imidazole and 10 mM calcium chloride and 5% (v/v) of DMSO buffer (pH 6.8) , 3= sample in 20 mM imidazole and 10 mM calcium chloride buffer (pH 6.8) filtered immediately after processing, 4 = sample in 20 mM imidazole and 10 mM calcium chloride and 5% (v/v) of DMSO buffer (pH 6.8) filtered immediately after processing

Conclusion

The separation of proteins by size exclusion chromatography from raw skim milk from individual cows was successful in isolating native casein micelles. Whey proteins were found associated with the casein micelles in the elution fractions. The results of this work suggest that the whey proteins associate with the casein micelle during milk production. The presence of whey proteins in association with native casein micelles is a significant finding since it stands contrary to the previously thought composition of the casein micelles. The pH value of the sample had no effect on the micelle-whey protein ratio when tested under reducing conditions. The samples with a pH of 5.0, when tested under non-reducing conditions, showed no whey proteins bands on the SDS-PAGE gels. This indicates that the whey proteins form very strong interactions with the caseins at those pH values. A possible avenue for the association is the highly porous structure of the micelle. However, we were unable to exploit the porosity and binding properties of the micelle to associate a hydrophobic peptide with the native micelle. This may indicate that the micelle has selective binding properties, with some compounds or molecules being more readily bound than others.

Acknowledgements

The authors wish to acknowledge the staff at the Little River Animal and Environmental Unit of the University of Tennessee AgResearch East Tennessee Research & Education center for their assistance in this work.

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CHAPTER IV

IMPROVING CASEIN STABILITY AT LOW PH VIA ETHANOL ESTERIFICATION BY SHIFTING THE ISOLECTRIC POINT OF THE PROTEINS

This chapter is a lightly revised version of a paper by the same title to be submitted to *Food Hydrocolloids* by Raymundo Trejo, Sasha Wilkinson, and Federico M. Harte. The use of "our" in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the experimental work, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the writing.

Abstract

Casein proteins are unstable when the pH of an aqueous phase is ≤ 4.6, limiting the use of skim milk powders for fortifying low pH fruit juices, soft drinks, and sports drinks. The esterification of proteins has been shown to cause a shift in their isoelectric point towards more alkaline values; which results in an increase in solubility at lower pH values. In fluid milk, casein is found in the form of micelles, which are held together by a combination of hydrophobic and colloidal calcium-protein interactions. These interactions may prove to be an obstacle to effective esterification of the individual proteins. This obstacle could be overcome by disassociating the casein micelles prior to esterification, which could be achieved by heating a milk sample containing 60% (v/v) ethanol and lowering the pH of the sample.

In this work, we evaluated the effect of pH prior to high temperature ethyl esterification of milk proteins in terms of casein and whey protein dispersibility and stability in fruit juice as a model food system. After pH adjustment and heat treatment at 121^{o}C for 15 min, the dispersions were lyophilized and the resulting powders dissolved in pH-adjusted white grape juice (pH 2 to 5). The solutions were allowed to settle and the protein content, protein composition, and particle size of the supernatant were measured. Changes in the molecular weight of the caseins were evaluated via Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). We detected an increase in the molecular weight of the caseins after esterification. The milk proteins esterified at pH values of 10 to 5

behaved in a similar fashion as the skim milk powder control. The milk proteins esterified at pH values of 2 to 4 showed improved stability and protein content in white grape juice adjusted to pH < 4. Both casein and whey proteins were found present in the supernatant of juice containing those powders. Also, particles of 400-600 nm in size were measured in the supernatant of the samples, which could indicate that the caseins are in micelle form. Lowering the pH of skim milk-ethanol solutions to 3 and 4 improves casein micelle dissociation and casein esterification resulting in milk powders that are more readily dispersed in low pH than non-esterified milk proteins.

Introduction

Milk proteins are an important ingredient to the food industry and caseins account for approximately 80% of total protein content in milk. In fluid milk, caseins are found in the form of micelles that are stable to relatively high temperature (121^{o}C) and pressure (<200 MPa) (Fox and Brodkorb, 2008). However, micelles aggregate and precipitate at pH values at or below the isoelectric point of casein (4.6) limiting their application in high acid liquid products, such as soft drinks and fruit juices. In fact, the most commonly utilized dairy proteins for the enrichment of high acid beverages are whey proteins.

The solubility of the caseins slowly increases as the pH value decreases (Chobert et al., 1990). Therefore, skim milk powder dissolved in a medium with a pH value close to the isoelectric point results in large amounts of precipitation of the casein proteins. Improving the solubility of caseins at pH values near the isoelectric point would allow for their use towards protein enrichment of acidic liquid products. Esterification of milk proteins has been proposed as a means to overcome isoelectric precipitation by shifting the isoelectric point of the protein towards more alkaline values (Chobert et al., 1990, Sitohy et al., 2001). Chobert et al., suggested inducing positive charges on β-casein by adding positively charged functional groups, and Sitohy et al. proposed the esterification of milk proteins to block negative charges, increasing the net positive charge of the protein thus raising its isoelectric point (Sitohy et al., 2001).

Figure 4.1 shows the proposed esterification reaction involving an amino acid and ethanol. Since the reaction requires an available carboxylic acid functional group, esterification would take place at the aspartic and glutamic acid functional groups, and at the C-terminus end of the protein. As seen in **Figure 4.2**, there are several locations on the amino acid sequence of casein proteins where the esterification reaction may take place. The number of possible esterification sites and expected change in the molecular weight of the resulting milk proteins when fully esterified can be seen in **Table 4.1.**

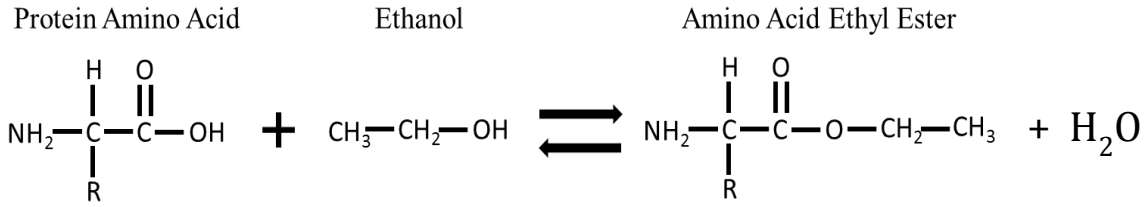

Figure 4.1. *Esterification reaction involving a protein amino acid and ethanol.*

Table 4.1. *Number of possible esterification sites and the expected increase in the molecular weight of the milk proteins when fully esterified.*

	α_{s1}-CN	α_{s2}-CN	κ-CN	β-CN	β-Lg	α-La
Aspartic Acid	7	4	3	4	10	9
Glutamic Acid	25	24	12	19	16	8
C-Terminus	1	1	1	1	1	1
Total Esterification Sites	33	29	16	24	27	18
MW	23623	25238	19006	23988	18227	14176
MW_E	24549.31	26052.03	19455.12	24661.68	19034.89	14681.26
ΔMW	926.31	814.03	449.12	673.68	757.89	505.26

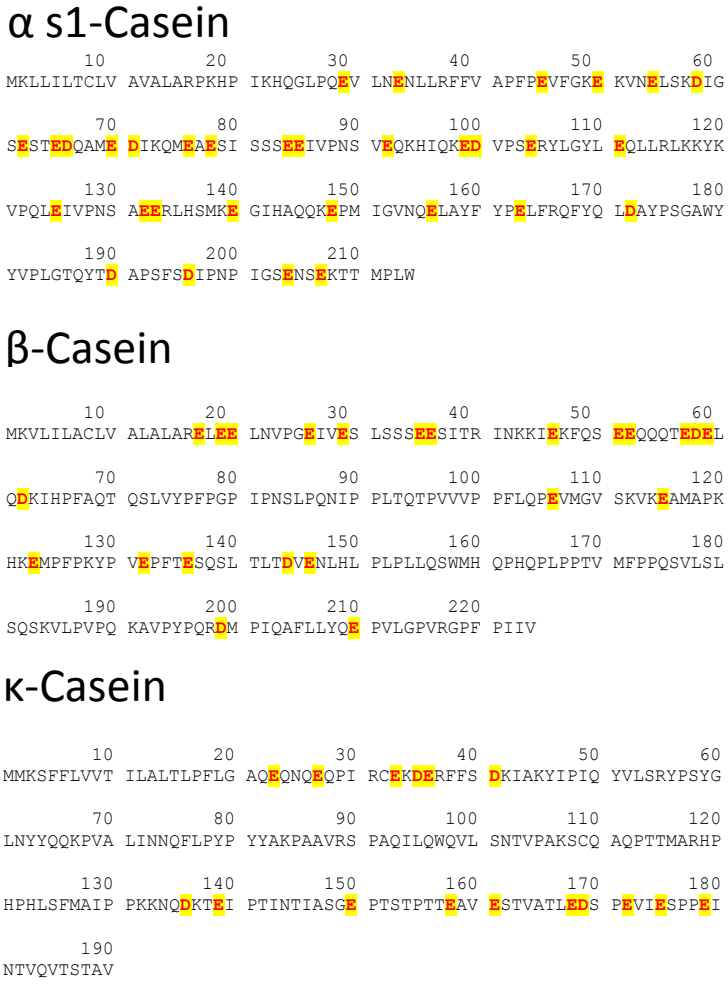

Figure 4.2. *Amino acid sequences of alpha s1, beta, and kappa caseins showing the aspartic and glutamic acid functional groups where the esterification reaction can occur (available groups are highlighted). (Source: the National Center of Biotechnology Information Protein Blast database http://blast.ncbi.nlm.nih.gov).*

The esterification reaction was demonstrated in isolated β –casein (Sitohy et al., 2001). However, due to hydrophobic and colloidal calcium-protein interactions between the caseins in the micelles, the native micellar state of caseins in fluid milk could hinder esterification of all casein proteins. The ethanol-mediated and heat-induced dissociation of the casein micelles has been previously reported (O'Connell et al., 2001, Zadow, 1993) and it was shown that the pH of milk and ethanol solution affects micellar disassociation (Roach, 2006, Ye Ran, 2012). The objective of this study was to evaluate the effects of pH of ethanol/skim milk solutions on the dissociation-esterification of caseins and the effect of esterification on the functional properties of casein micelles in a food system (white grape juice). Our fundamental hypothesis was that by lowering pH prior to ethanol esterification, casein micelle dissociation would be induced and efficient casein esterification would be obtained. After dehydration and esterification of the caseins, introduction in a liquid medium would promote re-formation of the casein micelles with modified properties, such as improved solubility at pH values near the isoelectric point. The functional properties of these esterified protein powders were tested in a fruit juice to evaluate their performance in a food system.

Materials & Methods

Milk source & sample preparation

Commercial pasteurized skim milk (PSM) was obtained at a local grocery store. A 60% ethanol: water solution (v/v) was prepared and pasteurized skim milk was added to obtain a final ratio of 1 ml of PSM for every 9 ml of ethanol solution. In order to disassociate the casein micelles, the sample was heated to 30^{o}C and the pH adjusted using 1 N HCl or 1 N NaOH to one of the following values: 2, 3, 4, 5, 6, 7, 8, 9, and 10. The samples were then autoclaved for 15 minutes at 121 ^{o}C to remove the bulk of the ethanol. Following autoclaving, the samples were stored overnight at -40 ^{o}C and then lyophilized. The resulting ethanol modified powders (EMP) were inspected for physical appearance, and stored in vacuum and with reduced humidity at a temperature of 4 ^{o}C. As a control, PSM was lyophilized to produce pasteurized skim milk powder (SMP) and stored under the same conditions as the EMP samples.

The powders, at a concentration of 0.5 g/ml, were dispersed in commercial pasteurized white grape juice (native pH 2.7) with constant agitation. In order for the powders to dissolve, the alkaline samples (EMP 7 – 10) were heated to 30 ^{o}C for 1 hour at the start of the agitation. The rest of the samples were not heated. All of the samples were stirred overnight. The pH of the fortified juice samples was then adjusted to 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0, using 1N HCl. Aliquots of the samples at the different pH values were collected and stored at 4 ^{o}C for 48 hours prior to stability determination and further analysis. The procedure was replicated three times.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)

The samples were prepared and processed using a slightly modified version of the method outlined by Fedele et al. (Fedele et al., 1999). Skim milk powder, EMP6, EMP5, EMP4 and EMP3 were dissolved under constant agitation in distilled deionized water at a rate of 0.04 g per ml. 100 µl of the dissolved milk powder was dissolved in a 0.1 % (v/v) trifluoroacetic acid (TFA) solution. MALDI-TOF measurements were done with an Applied Biosystems Voyager-DE™PRO Biospectrometry Workstation (Life Technologies Corp., Carlsbad, CA). Ions formed by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) were accelerated at 25 keV. The UV laser light (50µJ) was focused on the samples using a variable focal diameter of 50-100 µm. For all the samples, sinapinic acid (saturated solution in acetonitrile-water (50:50, v/v) was used as a matrix. Volumes of 1 µl of the sample solution were added to 24 µl of matrix solution. A 1 µl aliquot of the resulting mixture was deposited on the stainless steel sample holder and allowed to dry at room temperature before introduction into the mass spectrometer. Three measurements were done for each sample in order to verify the reproducibility and the mass accuracy, which was always in the range of 0.1-0.5%. External mass calibration was performed using the $[M+H]^+$ and $[M+2H]^{2+}$ ions of horse myoglobin at *m/z* 16925 and 8476, respectively.

Particle size analysis

An aliquot sample from the top layer of the supernatant was collected using a pipette. To obtain an optimal concentration for particle size measurement, the sample was diluted with deionized (DI) water. The samples were placed in a quartz cuvette, and particle size measurements via dynamic light scattering were conducted utilizing a Delsa Nano C particle size analyzer (Beckman Coulter, Brea, CA). The Delsa Nano was set up to record 50 measurements with a 50 μm pin hole aperture. Average micelle particle size was calculated utilizing a refractive index of 1.33, the CONTIN method for particle size distribution, and the cumulant method for dynamic light scattering particle size (Frisken, 2001, Koppel, 1972). .

Three repetitions of three measurements were performed and the average of the three repetitions was calculated.

Protein content analysis

The supernatant was removed via careful pipetting, and filtered through Miracloth to prevent any large particles from being transferred along with the supernatant. The total nitrogen of the supernantant samples was measured by the Dumas combustion method (ISO 14891/IDF185) using a TruMac® N analyzer (Leco, St. Joseph, MI), and the protein content was calculated utilizing a conversion factor of 6.38. Due to a limited amount of powder samples, two repetitions with two measurements were conducted and the average of the repetitions was calculated.

SDS Polyacrylamide gel electrophoresis

A sample loading buffer was prepared using 0.5 M Tris-HCL pH 6.8, glycerol, 10% (w/v) SDS, β-mercaptoethanol, 0.5% (w/v) bromophenol blue in water (FisherScientific, Fair Lawn, NJ). A 100 µl aliquot sample from the top portion of the supernatant for each of the pH samples was combined with 400 µl of the loading buffer and vortexed. The samples were heat denatured for 5 minutes at 95 °C prior to electrophoresis. SDS-12%PAGETris-HCl ready gels were used for electrophoresis (12 well precast gel, Biorad, Hercules, CA). Sample volumes of 18 µl were loaded into wells and electrophoresis was run at 200 volts for 38 minutes. Gels were stained using a Coomasie blue stain and destained until the protein bands became visible. The gels were scanned and analyzed by densitometry using an image analysis software package (ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). All experiments were repeated three times.

Results and discussion

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)

Previous studies on the esterification of milk proteins showed that the changes in the molecular weight of the esterified proteins were too small to be detected via electrophoresis (Sitohy et al., 2001). Therefore, we required a more

sensitive method to be able to detect the minute changes in the molecular weight of the samples. Based on previous literature (Fedele et al., 1999), MALDI-TOF would be a suitable method for detecting the shift on molecular weight. As seen in **Figure 4.3**, there was a shift on the peak corresponding to the molecular weight of the caseins. Based on previous literature (Chobert et al., 1990, Mattarella et al., 1983, Sitohy et al., 2000, Sitohy et al., 2001), and the conditions present the most likely cause of this increase on the protein molecular weight is due to their esterification. Maillard reaction between the proteins and lactose could also result in an increase of the molecular weight of the caseins. However, the acidic conditions present in the samples tested would greatly limit or stop the reaction (Hans-Dieter Belitz, 2009). Furthermore, studies on the effects of the Maillard reaction of casein with dextran (Aminlari et al., 2005, Cardoso et al., 2011) have shown that the increase in molecular weight of the caseins is much larger than the one detected by MALDI-TOF. The changes in the molecular weight of casein seen with Maillard reaction are large enough that they can be detected via SDS-PAGE.

The increase on the molecular weight of the proteins became more significant as the pH of esterification was reduced. However, the EMP3 sample did not show an obvious peak for any of the caseins. This is most likely the result of protein hydrolysis due to the lower pH of the sample and the high temperature of the process (Hans-Dieter Belitz, 2009, Fennema, 2007). The higher degree of esterification seen on the EMP4 sample indicates that the pH value that is optimal for obtaining the desirable balance of larger number of caseins available

for modification versus having major protein hydrolysis is found near a pH of four. Further experiments could help determine the pH value at which this balance is achieved.

Physical properties of the esterified milk powders

The pH at which the samples were modified had an effect on the physical appearance of the lyophilized powders. **Figure 4.4** shows the lyophilized powders for each of the pH values. The sample that was esterified at a pH 2.0 (EMP2) produced dark purple to red pigmented aggregates in the powder. The darker color was probably due to Maillard browning. The aggregates and red coloration were only found in these samples. The samples modified at a pH of 3.0 (EMP3) and 4.0 (EMP 4) produced free flowing powders. The samples modified at pH 5.0 (EMP5), 6.0 (EMP6), 7.0 (EMP7), 8.0 (EMP8), and 9.0 (EMP9) produced flakes instead of a loose powder. The flakes were thicker and they compacted into sheets in the EMP6 sample. The EMP 10 samples also produced free flowing powders. As the pH of esterification increased from 8.0 to 10.0 (EMP8, EMP 9, EMP10), the powdered samples became increasingly darker in color. This was most likely the result of Maillard reaction between the reducing sugar lactose and proteins in alkaline conditions (Hans-Dieter Belitz, 2009).

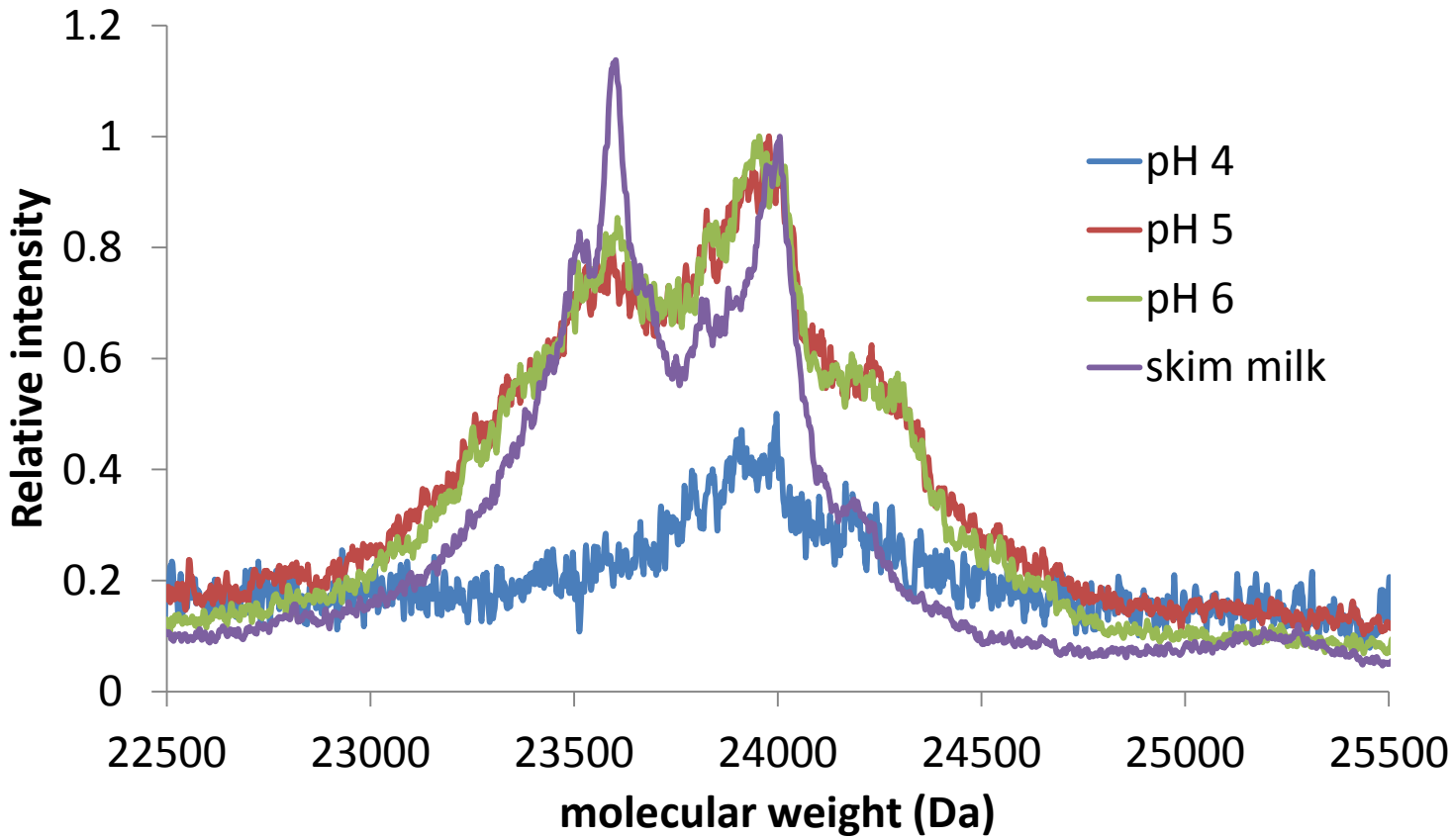

Figure 4.3. *Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) relative intensity of the casein proteins for skim milk powder, milk powder esterified at pH of 6.0 (EMP6), milk powder esterified at pH of 5.0 (EMP5), and milk powder esterified at pH of 4.0 (EMP4).*

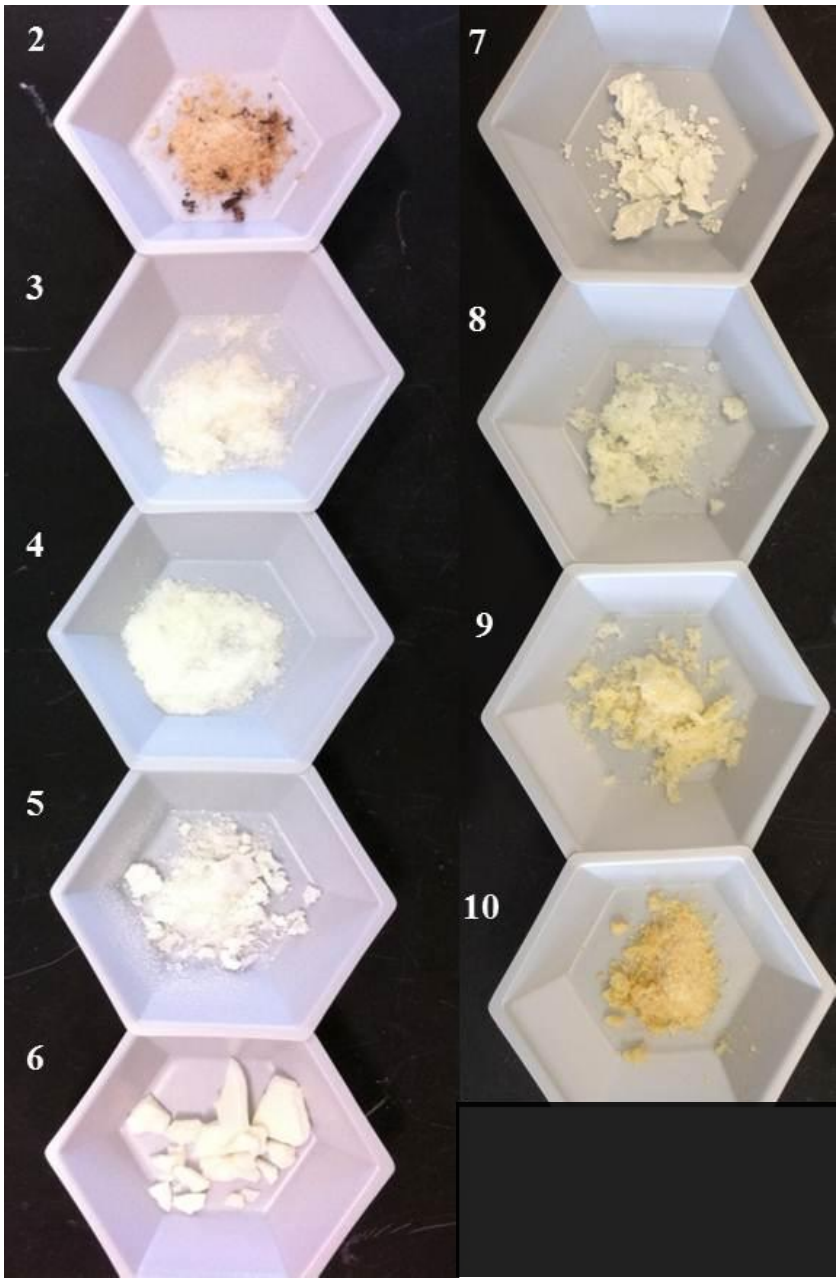

Figure 4.4. *Lyophilized powders of treated milk powders. The number indicates the pH of the samples before thermal processing.*

The dispersion and stability of the different milk powders in grape juice is shown in **Figure 4.5**. Milk powders modified at low pH (EMP2, EMP3, and EMP4) were stable after 48 hours in grape juice with a pH value of 3.0 or lower, and EMP2 and EMP3 were stable in the grape juice with a pH up to 3.5. In all cases where stable dispersions were obtained, the turbidity of the juice was increased due to the addition of the milk powders. The color change was most noticeable in the juice containing EMP2, where the juice gained a reddish color and became darker than the other samples. The lyophilized commercial skim milk powder control was not soluble and precipitated at all the pH values tested. Eriksen (1991) observed limited casein solubility when skim milk powder was suspended in liquids with pH below 4.0, and the solubility decreased further once the pH dropped below 3.0 (Eriksen, 1991). Eriksen reported that the solubility of casein in acidic pH values increased via hydrolysation of the caseins via enzymatic treatment. However, the hydrolyzed proteins had a bitter flavor. This made casein hydrolysates unacceptable as food ingredients in order to fortify fruit juices (Eriksen, 1991). By shifting the isoelectric point of the caseins, the esterification method being presented here could offer a solution to this obstacle. Further research on the sensory properties of the esterified milk proteins is needed in order to establish the commercial feasibility of this treatment.

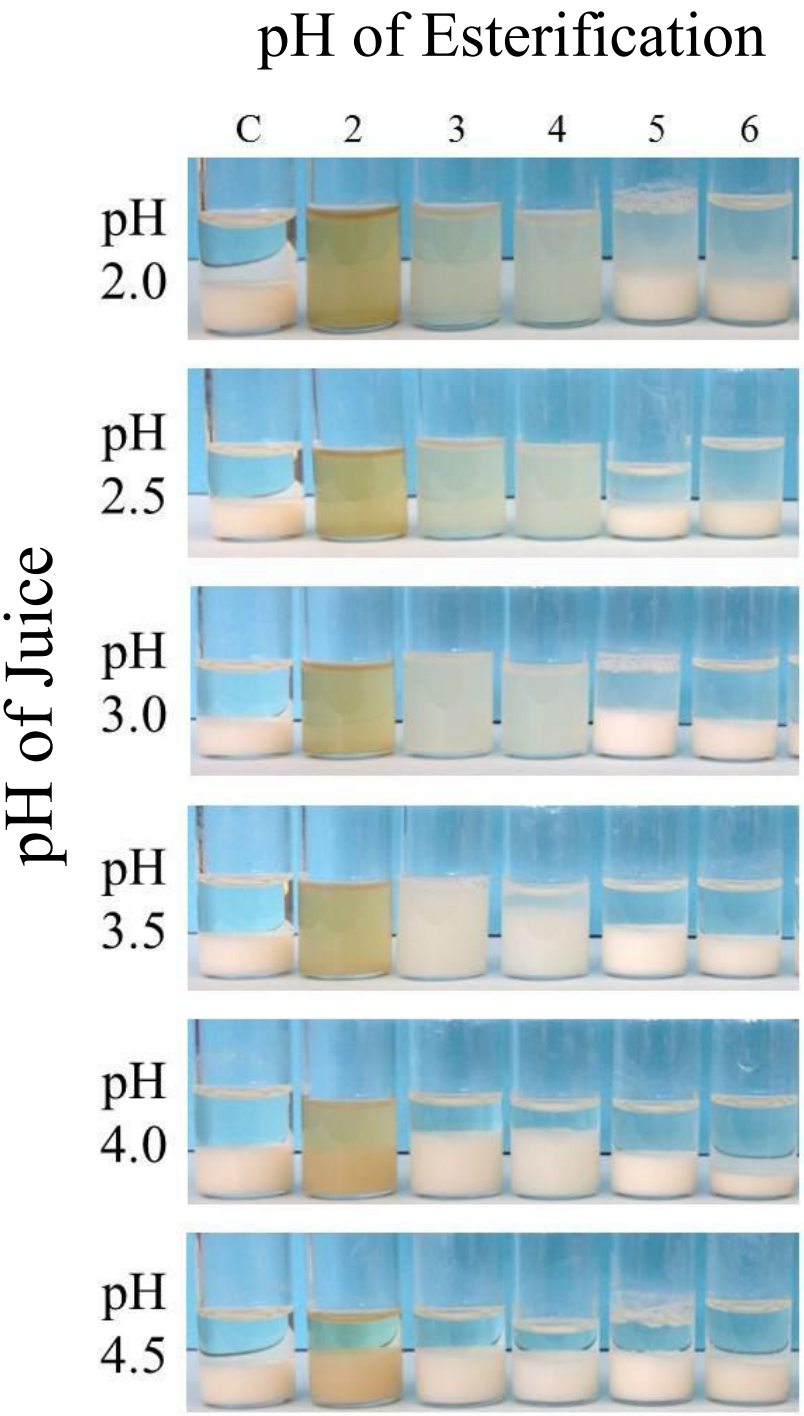

Figure 4.5. *Visual stability of esterified milk powders and pasteurized skim milk powders in white grape juice. Images taken 48 hours after dispersion at different pH values. C=Pasteurized skim milk.*

Particle size analysis

The lyophilized skim milk powder precipitated at all pH values, and exhibited large particles in the supernatant liquid. As seen in **Figure 4.6**, the size of the particles in the supernatant decreased when the pH of the grape juice was raised above 4.5. However, as seen in **Figure 4.7**, the average size of the particles in the supernatant was still larger than that of the casein micelles seen in fluid milk. The effects of pH on the size of casein micelles were studied by Liu et al. (Liu and Guo, 2008). In their work, they observed that casein micelles reconstituted from milk powder had a similar size to those found in native pH. This is inconsistent with our observation, since at pH values above 3.0, all of the esterified milk powders also had large particles in the supernatant. This larger size of the casein micelles was also reported by O'Connell et al., in casein micelles reformed from heated mixtures of milk and ethanol (O'Connell et al., 2003). In their work, they found that reformed micelles had a wide size distribution, with diameters that ranged in size from 1000 to 4000 nm. This was also seen in our results. However, at pH values below 3.0, EMP4 and EMP3 had a smaller apparent average particle size. The apparent average particle size in these juice samples, 400 – 600 nm, indicated that the caseins were probably dispersed in micelles of comparable size range as those found in fluid milk. These were also the samples where the majority of the milk proteins remained in suspension.

From a nutritional perspective, enriching fruit juice with a milk protein powder containing micellar casein in large quantities alongside the whey proteins results in a more nutritious product. The whey proteins have a fast digestion rate

while the caseins have a slower rate of digestion (Boirie et al., 1997). The slower rate of digestion facilitates a more efficient amino acid uptake into the body. By providing proteins with both a fast and slow digestion rate, enriching fruit juice with these modified milk proteins would provide an advantageous balance of protein and amino acid uptake into the body. Therefore, whey proteins alone do not provide the same nutritional value as casein or total milk proteins (Lacroix et al., 2006).

Protein content analysis & SDS Polyacrylamide gel electrophoresis

As initially expected, the addition of milk powder, both modified and SMP, resulted in higher protein content than the native protein content of white grape juice (**Figure 4.8)**. However, the higher protein concentration on the juice mixed with the skim milk powder (SMP) was primarily due to whey protein content and to a lesser extent dissolved caseins. Milk powder esterified at pH 5.0 to 10.0 (EMP 5-10), did not show higher protein content than the SMP control. When the white grape juice had a pH value higher than 4.0, none of the treatments had a higher protein content than the SMP control. However, in juices with pH of 3.5 and below, there was a higher casein protein concentration on the juice samples containing milk powder esterified at pH 2.0 to 5.0 (EMP2, EMP3, EMP4, and EMP5). These higher protein contents and the bands found in the SDS-PAGE gels show that these treatments were effective in maintaining a larger proportion of casein proteins in suspension at pH below 3.5.

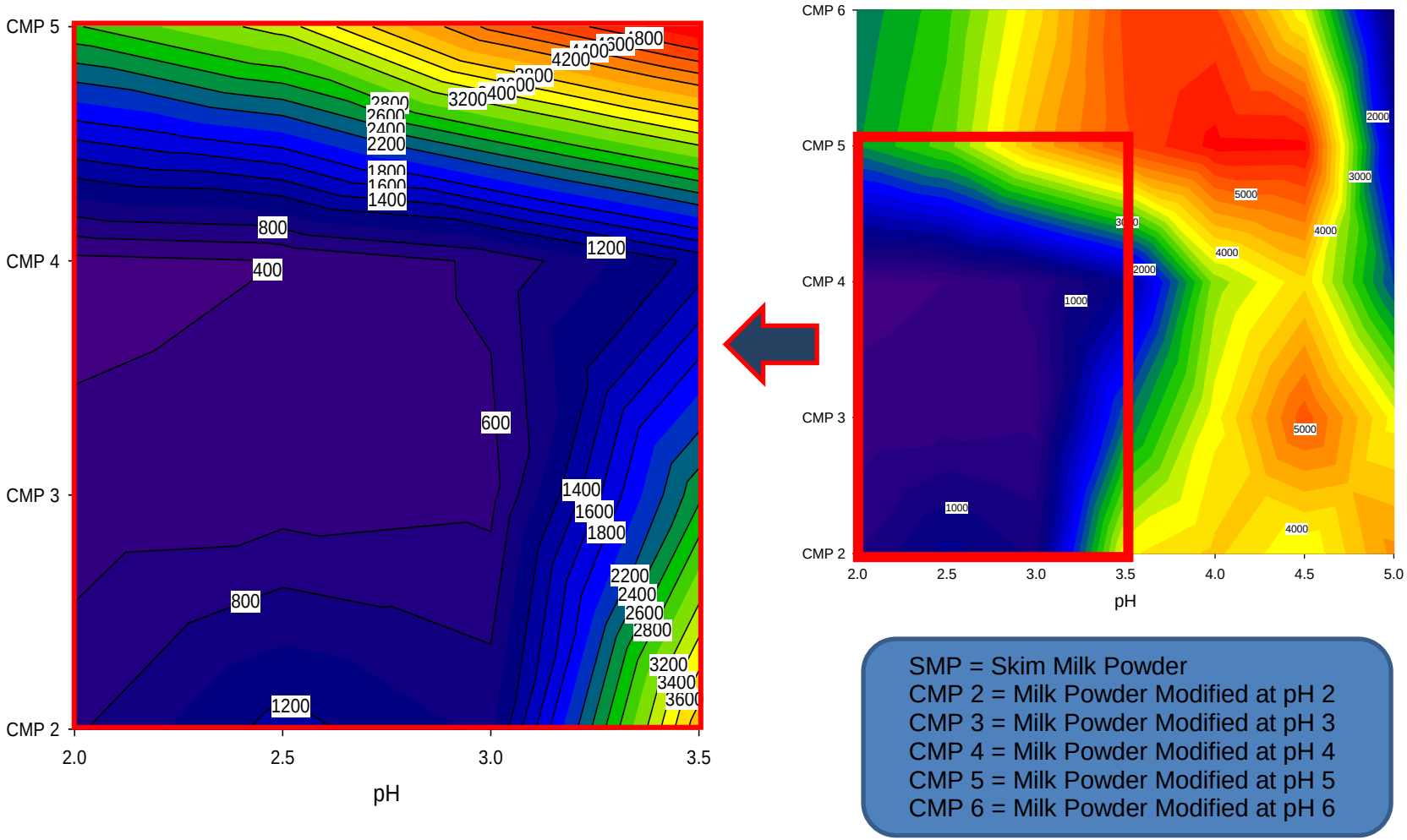

Figure 4.6. *Average particle size in the supernatant of white grape juice mixed with esterified milk powders and pasteurized skim milk powders (SMP) at different pH values.*

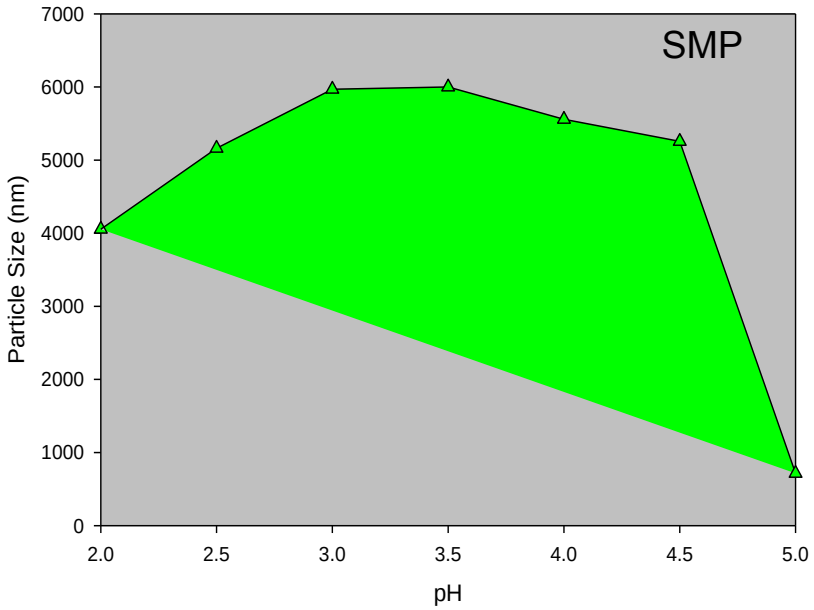

Figure 4.7*. Average particle size in the supernatant of white grape juice mixed with pasteurized skim milk powders (SMP) at different pH values.*

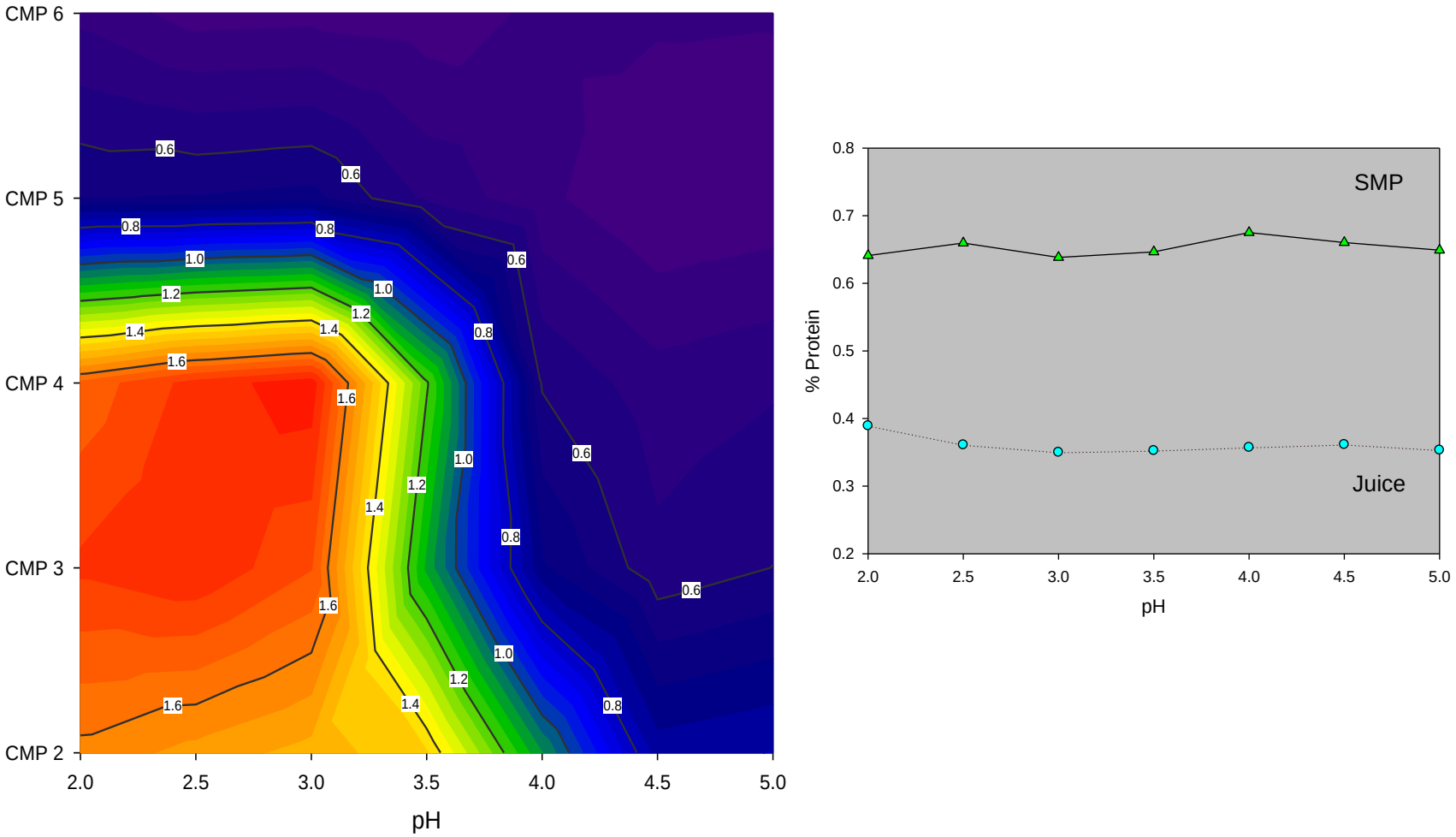

Figure 4.8. *Protein content percentage in the supernatant of white grape juice mixed with esterified milk powders, pasteurized skim milk powders (SMP), and white grape juice at different pH values.*

As previously reported, the increased solubility of the caseins is due to the shifting in the isoelectric point towards the alkaline scale (Chobert et al., 1990, Sitohy et al., 2000, 2001). This in turned increased the amount of soluble casein at a pH value of 3.5. EMP 3 and EMP 4 produced samples that contained more than 1.6 percent protein. In a practical application, a protein fortified fruit juice prepared with either one of those powders and with a serving size of 10 fluid ounces / 296 ml would contain 4.73 grams of milk protein per serving. The majority of the proteins in this product would be caseins, which have been shown to have a higher nutritional value than the whey proteins.

Figure 4.9 shows the SDS-PAGE band patterns of juice samples at different pH values. At pH values of 2.0 and 2.5, casein bands were visible in the gels for most of the treatments and the controls. However, the esterification of the caseins resulted in larger amounts of casein being found in the samples containing EMP 2, 3, 4, 5, 6, and 7 at pH values of 3.0 and below. In particular, EMP3 and EMP4 showed the most casein bands at those pH values. At pH 3.5, only EMP 4 and EMP5 showed significant casein bands in the gels. Although EMP2 contained casein proteins at low pH values, due to the sample's pH and properties, there were no clearly visible casein bands in the gel. The presence of small amounts of casein at pH values below 2.5 could be due to the self-aggregation of β-casein into micelles (Portnaya et al., 2008). However, the 20 - 25 nm average micelle size reported by Portnaya et al., was much smaller than the average particle size recorded in our work.

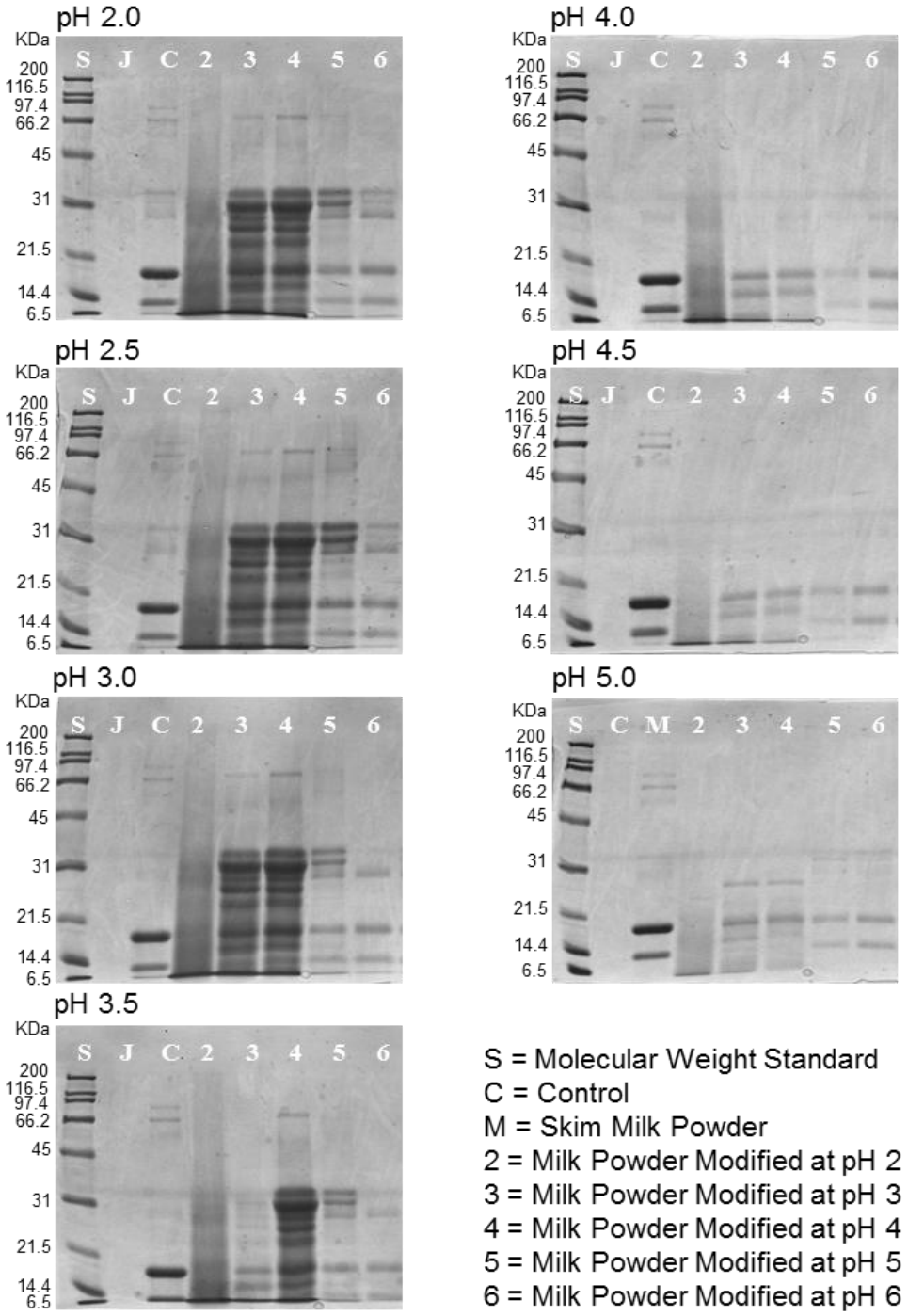

Figure 4.9. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the supernatant of white grape juice mixed with esterified milk powders and pasteurized skim milk powders at different pH values.*

Figure 4.10 further illustrates the noticeable increase in the solubility of caseins esterified at pH values of 3.0 and 4.0. These powders showed a higher level of solubility than the SMP and all of the other treatments when the pH of the juice was set between 2.0 and 4.0. However, it should be noted that the higher temperatures utilized in the protein esterification also caused some protein degradation, as can be seen in the SDS-PAGE. As previously mentioned, the most likely cause for the protein degradation is the partial hydrolysis of the proteins due to the low pH values and high temperatures involved in the treatments (Hans-Dieter Belitz, 2009, Fennema, 2007). Further studies could determine the optimum temperature that would provide an acceptable balance between protein esterification and protein degradation.

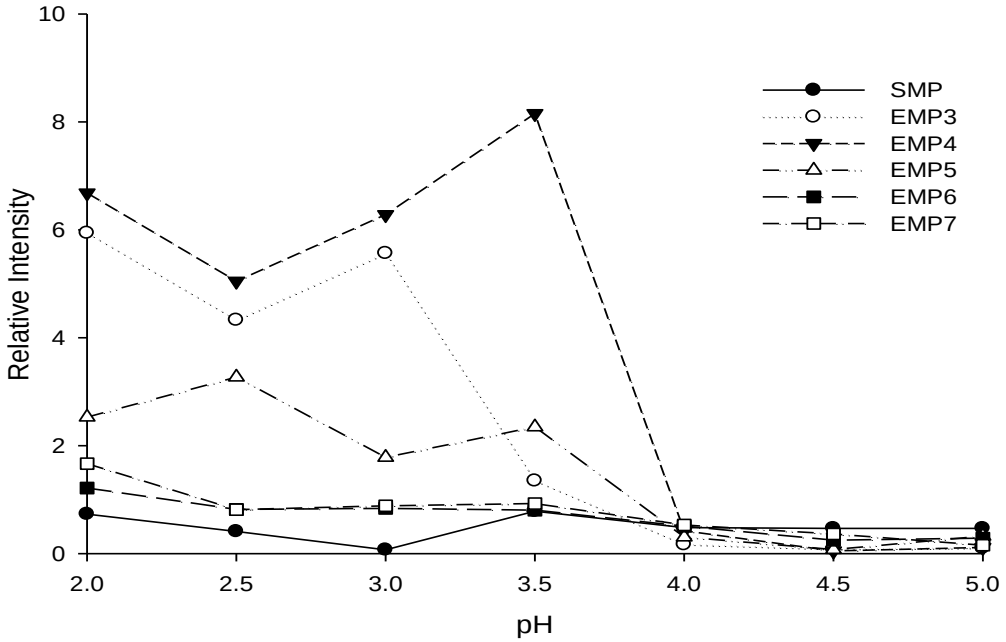

Figure 4.10 *Relative intensity of the casein bands shown in the SDS-PAGE across the pH values evaluated.*

The ethanol mediated chemical modification of caseins at pH values between 2.0 and 4.0 is an effective method to improve the solubility of caseins in high acid liquids. The caseins were soluble at pH values of 3.5 and below. The apparent size of the particles in the juice at pH below 3.5 would indicate that the casein proteins were found forming micelles. The suggested pathway for the modification of the isoelectric point of casein micelles by esterification is shown in **Figure 4.11**. The addition of ethanol promotes a temperature and pH dependant casein swelling of native casein micelles. Lowering the pH of the ethanol / milk solution to <5.0 promotes micellar dissociation into single casein protein monomers (Roach, 2006, Ye Ran, 2012). At this point, increasing the temperature favors ethyl esterification of individual proteins and partial ethanol volatilization. In the final step, lyophilization removes water and remaining ethanol and favors micellar reformation. Once the dehydrated esterified caseins are reintroduced into a liquid medium, both the esterified and unmodified caseins will interact with the calcium phosphate present in the powder and drive the self-association of the micelles. This would produce reformed casein micelles; which, due to their esterified casein components would have modified physicochemical properties, such as improved solubility at pH values near the isoelectric point.

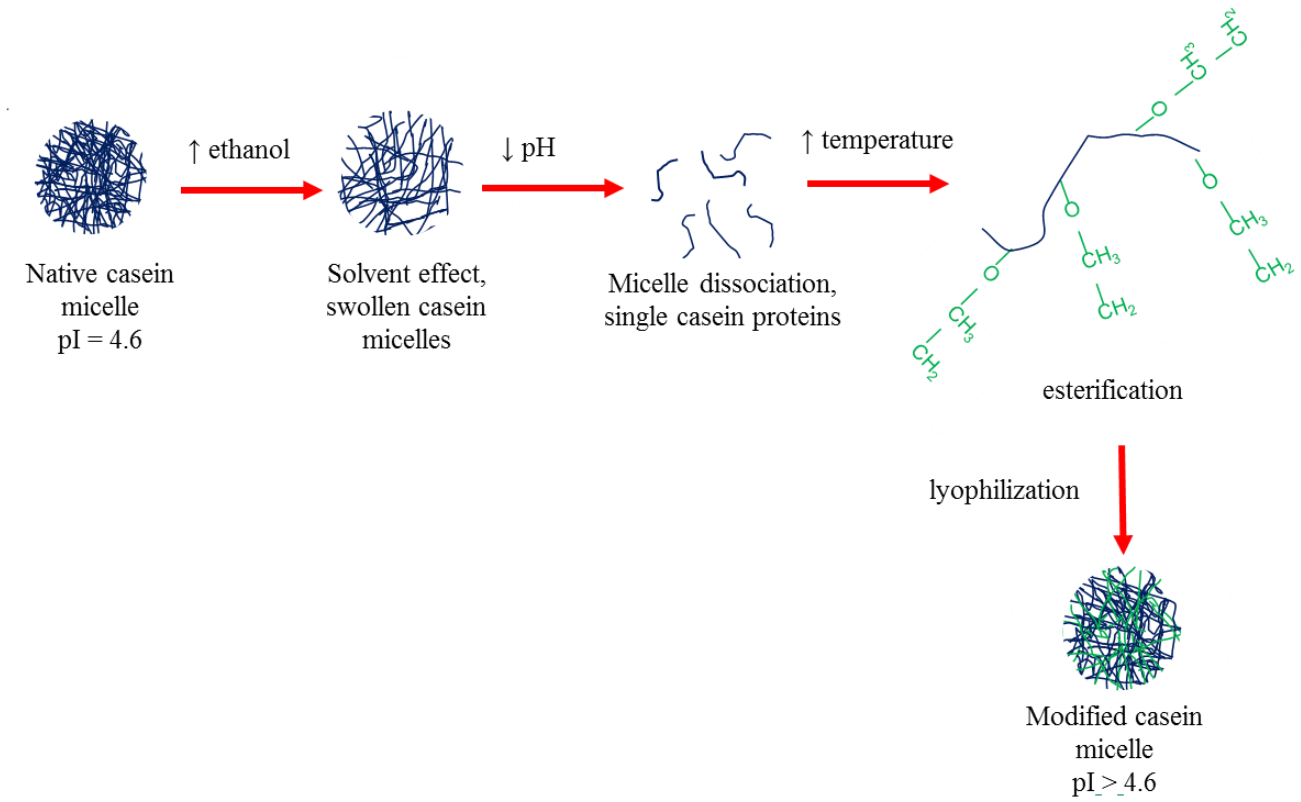

Figure 4.11. *Suggested mechanism for the change of casein micelles isoelectric point by ethyl esterification of casein proteins.*

Conclusion

The esterification of caseins by shifting the isoelectric point of the proteins, improves their solubility at pH values near the isoelectric point of the native proteins. By adjusting the pH of a skim milk and ethanol solution, it is possible to disassociate the casein micelle and improve the rate of esterification. According to our findings, a milk-ethanol mixture with a pH value of 4.0 results in a higher rate of esterification without any great loss of protein to hydrolysis. Once the esterified milk powders are rehydrated, the esterified caseins interact with non-modified caseins to produce modified casein micelles that show a better solubility at pH values ≤ 3.5. Improving the solubility of milk proteins in acid conditions would permit their use in acid applications such as soft drinks and fruit juices, resulting in a more nutritious product.

The esterification reaction attaches ethyl groups to the proteins by removing the hydroxyl from the alcohol. Therefore, this method can potentially be used to attach other functional groups to the proteins, as long as they have a hydroxyl group. A candidate for further studies could be the sugar alcohol xylitol; which contains 5 hydroxyl groups. By utilizing the described method, it would be possible to attach xylitol to the caseins to produce modified casein micelles with different physicochemical properties that can be of interest to the food industry.

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CHAPTER V

THE EFFECTS ON THE PROPERTIES OF YOGURT OF CASEIN MICELLE MODIFICATION BY A LOW TEMPERATURE STEP IN THE MANUFACTURE PROCESS

This chapter is a lightly revised version of a paper by the same title to be submitted to the *Journal of Dairy Science* by Raymundo Trejo, Sasha Wilkinson, and Federico M. Harte. The use of "our" in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the experimental work, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the writing.

Abstract

Dairy products are an important economic commodity, and the popularity of yogurt as a dairy product is well known. In fat-free yogurt, it is difficult to obtain a firm gel product that is resistant to syneresis. Manufacturers employ stabilizers, like gelatin, starches, or pectin, to prevent syneresis. It has been shown that at low pH and temperature, β-casein and calcium move out of the casein micelle. Introducing a cold step into the fermentation process of yogurt when the pH of the milk is ~5.2 would stop the fermentation and allow the caseins and calcium to move out of the micelle. This would result in smaller micelles which in turn would result in a tighter gel matrix. The compact gel matrix should provide better whey holding capacity (WHC) and a firmer product. Pasteurized skim milk was fortified with 2% (w/v) skim milk powder and inoculated with a yogurt culture. The samples were batch fermented at 40 ^{o}C until pH ~5.2 and held at 5 ^{o}C for 30, 60, 90, and 120 minute intervals before being re-heated and allowed to ferment to a pH of 4.6-4.5. The viscosity of the acidified milk was measured at the starting point, prior to the cold step, and at the different intervals in the cold process. The dissociated β-casein content of the samples at the different cold step intervals was identified via SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). The WHC and the storage modulus (G') of the resulting yogurt samples were measured.

The viscosity measurements of the acidified milk samples showed that it had a weak gel structure at pH 5.2. The viscosity of the samples returned to

values similar to fluid milk after the temperature was reduced. The amount of dissociated β-casein found in the samples increased as the length of the cold step also increased. The WHC and G' values for the yogurt samples that were held for 60, 90, and 120 minutes were statistically different and higher than the controls.

As the temperature and the pH decreases, β-casein and calcium migrate out of the casein micelle. Once the temperature is increased to the fermentation range, the liberated β-casein forms small micelles; which combined with the smaller original micelles form a more compact gel structure upon further acidification. The compact structure results in a stronger gel with good WHC without the need for the addition of stabilizers or gums.

Introduction

Dairy products, such as yogurt, are an important economic commodity. Yogurt is usually produced by fermenting milk with the bacteria *Streptococcus thermophiles* and *Lactobacillus delbrueckii* sp. *bulgaricus*. These bacteria commonly referred to as lactic acid bacteria convert the lactose in milk into lactic acid. The lactic acid reduces the milk's pH value; which plays an important role in the gel formation (Peng et al., 2009, Sodini et al., 2004).

The physical attributes of yogurt (e.g., perceived viscosity and lack of whey separation) are important aspects for the quality and overall acceptance of the product by consumers (Lee and Lucey, 2010). In fat-free yogurt, it is difficult to obtain a firm gel product that is resistant to whey leeching out the gel matrix (syneresis) (Lucey and Singh, 1997). Manufacturers employ stabilizers, including gelatin, starches, or pectin, to prevent syneresis. Another approach to prevent or reduce syneresis is to increase the total solids content of the milk (Lee and Lucey, 2010). The addition of these components may be undesirable for consumers and add to the cost of the product. Therefore, the ability to produce a firm yogurt with a high whey holding capacity (resistance to syneresis) would be of interest to the industry. One such approach would take advantage of the physicochemical properties of the casein micelles, the main component in the yogurt gel matrix, in order to produce a stronger gel. It has been shown that at low pH and temperature, β-casein and calcium migrate out of the casein micelle; with the maximum dissociation of caseins from the casein micelle occurring when the pH

decreases from 5.6 to ~ 5.1 (Dalgleish and Law, 1988). Introducing a cold step into the fermentation process of yogurt when the pH of the milk is ~5.2 would stop the fermentation, and therefore maintain that pH value. According to Dalgleish and Law, the following processes are responsible for the migration of β-caseins from the micelle: the weakening in the hydrophobic interactions due to the reduced temperature, the dissolution of calcium and phosphate from the micelles. The migration of β-caseins and calcium would result in a size reduction of the original micelles. Once the temperature of the acidified milk returns to the fermentation temperature, the hydrophobic forces are increased and the free β-caseins can interact with the solubilized calcium to produce small aggregated micelles. By reducing the size of the micelles, it would be possible to produce a stronger gel matrix to provide better resistance to syneresis and a firmer product. The purpose of this work was to evaluate the effect of a cold step in the processing of yogurt, and its effect on the properties of the yogurt.

Materials & Methods

Milk source & sample preparation

Commercial pasteurized skim milk (PSM) was obtained at a local grocery store. The PSM was fortified with commercial skim milk powder to a concentration of 2% (w/v). The concentrated milk was heated for 30 minutes at 85 ^{o}C. The milk was then chilled in an ice bath and stored overnight at 4 ^{o}C. The milk was heated to 40 ^{o}C in 2000 ml batches prior to inoculation with freeze dried yogurt culture (YF-L901, CHR Hansen USA, Milwaukee, WI). The milk was

inoculated with 0.02% (w/v) yogurt culture and stirred for 10 minutes. As a control (Control-A), 200 ml aliquots of the inoculated milk were poured into containers and allowed to ferment in the container until it reached a pH of 4.5 - 4.6. The Control-A containers and remaining 2,000 ml batch samples were placed in a controlled temperature chamber set at 45 ^{o}C to ferment. When the pH of the samples reached approximately 5.2, 200 ml aliquots of the inoculated milk were poured into containers and promptly returned to the warmer. These samples (Control-B) and the Control-A samples were allowed to continue fermenting until reaching a pH value of 4.5-4.6. The remaining batch samples were removed from the warmer chamber and placed in an ice bath. The samples were cooled using a tubular heat exchanger (Model 00527.2, Exergy LLC, Garden City, NT) connected to a refrigerated water bath (Isotemp 3016, FisherScientific, Fair Lawn, NJ) set at a temperature of 0 ^{o}C and by submerging sanitized ice packs into the acidified milk. The acidified milk was pumped through the heat exchanger in a counter flow configuration using a peristaltic pump set at a flow rate of 1 liter per minute. The temperature of the samples was reduced from 45 ^{o}C to 4 ^{o}C within 30 minutes. The pH of the chilled samples was recorded at 5.2-5.1. The samples were kept recirculating through the heat exchanger in order to ensure that the temperature of the sample was maintained constant. The samples were held at 4 ^{o}C and 800 ml aliquots samples were removed after 30, 60, 90, and 120 minutes. The aliquots were warmed to 40 ^{o}C within 30 minutes of removal from the chilled conditions, poured into containers in 200 ml aliquots and the containers were placed into the temperature controlled chamber to

complete their fermentation (pH 4.5-4.6). After the fermentation was complete, the samples were chilled in an ice bath for 30 minutes and stored for two days at 4 ^{o}C prior to testing. All experiments were done in triplicate with two sub-samples per replication.

Whey Holding Capacity

The whey holding capacity of the samples was calculated utilizing a modified version of the method developed by Guzman-Gonzalez et al. (Guzman-Gonzalez et al., 1999). The yogurt samples (Y) were weighed, and then they were centrifuged at 600 x *g* for 15 minutes at 4 ^{o}C. The whey was then carefully removed by pipetting, and the samples were weighed again. The change in weight was equal to the amount of whey removed (W). The whey holding capacity (WHC) was calculated utilizing the following formula:

$$WHC = \frac{Y - W}{Y} x1000$$

All measurements were done in triplicate with two samples per replication. An analysis of variance (ANOVA) was carried out utilizing the SAS software package to determine the statistical differences between the treatments (SAS 9, 2010) and least squares means were separated using Tukey's significant difference test. Significance level was established at $\alpha = 0.05$.

Transmission Electron Microscopy

Samples from Control A, Control B, and 90 min treatment yogurts were prepared for transmission electron microscopy (TEM) after being cut into 2 mm^3 cubes.

The cubes were submerged in a 0.05 M 1,4-Piperazinediethanesulfonic acid (PIPES) buffer (pH 6.8) containing 1.25% gluteraldehyde and 2.00% paraformaldehyde and left at 4 ^{o}C for 12 hours for fixation. After three 10 minute washes in PIPES buffer, the cubes were dehydrated through a series of 10 minute washes in 30%, 50%, 70%, and 95% (v/v) ethanol in distilled water. Dehydration was completed with three 10 minute washes in 100% ethanol. The samples were washed in PIPES buffer, then post fixed in 2% buffered osmium tetroxide for 60 minutes. Samples were then dehydrated in a graded ethanol series before embedding the Spurr epoxy (Polysciences, Inc., Warrington, PA). Eighty nanometer sections of the fixed and cured yogurt gel samples were obtained using a Leica EM UC7 ultramicrotome (Leica Microsystems Inc., Chicago, IL). Images were taken with a transmission electron microscope in scanning mode (STEM) (Zeiss Auriga, Zeiss,Jena, Germany) at 30 KV: 20 pA. The images were analyzed by using an imaging software package (ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/)

Rheological Properties

The viscosity of the samples was measured utilizing a TA Instruments AR 2000 rheometer (TA Instruments, New Castle, DE) using a 40 mm 1^{o} aluminum cone geometry with a truncation gap of 30 μm. A continuous ramp test was done with a ramp shear rate from 0 to 120 1/s with duration of 30 seconds in linear mode at a temperature of 25 ^{o}C. A 1 ml sample of the inoculated milk (40 ^{o}C) was removed and the viscosity was measured. After the pH of the acidified milk

reached ~ 5.2, a 1 ml sample was removed and the viscosity was measured. After the temperature of the acidified milk was reduced to 5 ^{o}C a 1ml sample was removed and measured at the following intervals: 0, 30, 60, 90, and 120 minutes. The storage modulus (G') was measured utilizing a standard-size stainless steel 4-vane rotor (2.8 cm diameter) and a cylinder immersed height of 25 mm. Oscillatory testing was done within the linear region (0.01% strain) for 30 seconds with 6 sample points recorded at a single frequency of 1 Hz and a temperature of 4 ^{o}C.

All experiments were repeated three times with two sub-samples per repetition. An analysis of variance (ANOVA) was carried out utilizing the SAS software package to determine the statistical differences between the treatments (SAS 9, 2010) and least squares means were separated using Tukey's significant difference test. Significance level was established at $\alpha = 0.05$.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

A sample of the acidified milk (pH ~ 5.2) at 5 ^{o}C was removed at the following intervals: 0, 30, 60, 90, and 120 minutes. The samples were immediately ultra-centrifuged for 15 minutes at 4 ^{o}C (100,000 x *g*) in a Beckman Coulter Optima™ XL-80K Ultracentrifuge (Beckman Coulter, Brea, CA). A 2 ml sample supernatant was carefully removed from the middle of the supernatant and stored at 4 ^{o}C until the electrophoresis was performed .

A sample loading buffer was prepared using 0.5 M Tris-HCL pH 6.8, glycerol, 10% (w/v) SDS, β-mercaptoethanol, 0.5% (w/v) bromophenol blue in

water (FisherScientific, Fair Lawn, NJ). A 200 µl aliquot sample from the supernatant was combined with 300 µl of the loading buffer and vortexed. The samples were heat denatured for 5 minutes at 95 °C prior to electrophoresis. SDS-12% PAGE Tris-HCl Ready gels were used for the electrophoresis (12 well precast gel, Biorad, Hercules, CA). Sample volumes of 18 µl were loaded into wells and electrophoresis was run at 200 volts for 38 minutes. Gels were stained using a Coomasie blue stain and destained until the protein bands became visible. The gels were scanned and analyzed by densitometry using an image analysis software package (ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). All experiments were done in triplicate.

Results & Discussion

Whey Holding Capacity

Figure 5. 1 shows the whey holding capacity for the treatments and the two controls. There was a significant difference ($p < 0.05$) between Control-A and Control-B, with Control-B having the lowest WHC value. The low WHC values for Control-B were most likely the result of the breakage of the weak gel structure that was forming at the pH (~ 5.2) in which it was transferred to the smaller containers. As shown by Lucey et al. in yogurt, syneresis can be due to an increase in the rearrangements of the gel matrix, or it can be caused by damage to a weak gel network. This damage can occur by vibration, agitation or cutting of the gel network (Lucey et al., 1998).

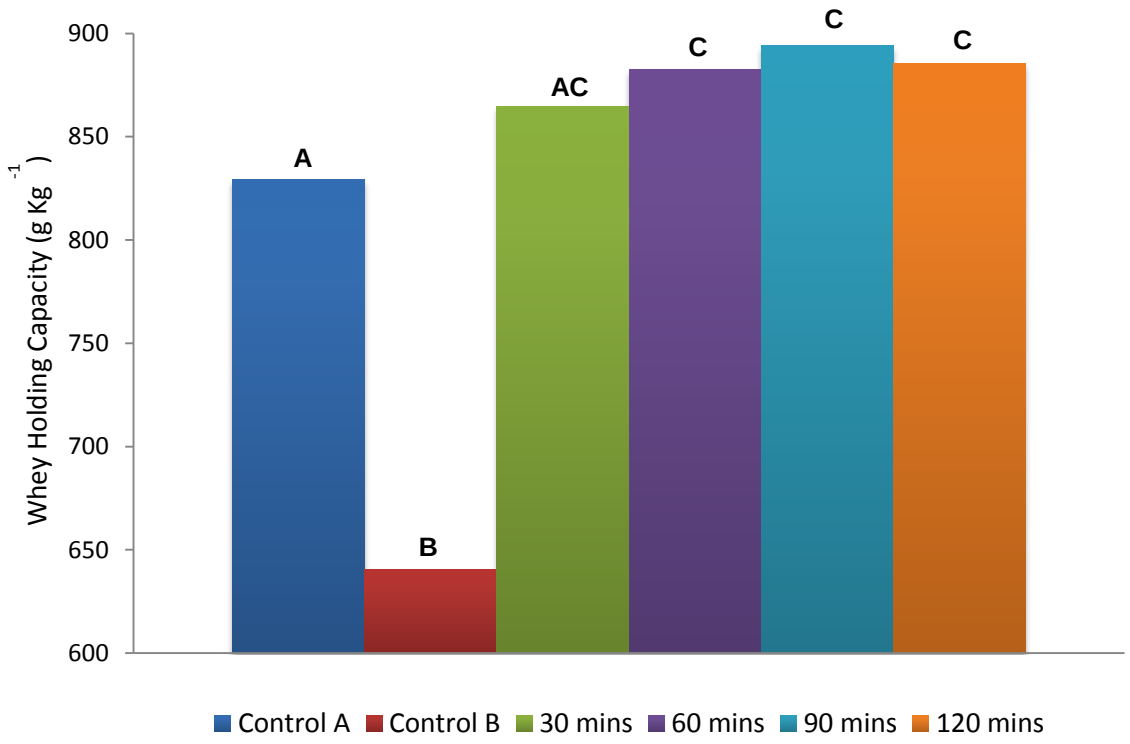

Figure 5.1. *Whey holding capacity of yogurt samples that were batch fermented at 40 °C until pH ~5.2 and held at 5 °C for 30, 60, 90, and 120 minute intervals before being allowed to ferment to a pH of 4.6-4.5. Control-A was fermented in individual containers. Control-B was placed in containers at pH 5.2 and 40 °C without a cold step. Different letters indicate a statistical difference between the values ($p < 0.05$).*

There was no statistical difference between Control-A and the sample that was chilled for 30 minutes. However, there was a statistical difference between Control-A and the treatments that were held at 5 ^{o}C for 60 minutes and higher. It has been demonstrated that at low pH (5.2) (Dalgleish and Law, 1988) and low temperature (≤ 5 ^{o}C) (Davies and Law, 1983, Walstra, 1990) β-casein migrates out of the casein micelle. Furthermore, at the pH values in which the samples were chilled (5.2 – 5.0), calcium and phosphate migrate out of the casein micelle (Dalgleish and Law, 1989). The solubilization of the calcium and phosphate is driven by the decrease in the pH values and it has been observed at various temperatures (Gastaldi et al., 1996) This β-casein and calcium migration results in a decrease of the size of the micelles. Once the temperature returns to the fermentation temperature, the liberated β-casein could form small micelles due to the hydrophobic forces being favored at higher temperatures. The gel network formed by the smaller micelles would have a better WHC than a much compact network.

This has been observed when comparing yogurt gels formed with heated and unheated milk. The whey was retained more effectively in gels made with heated milk, which had a casein matrix composed of small compartments, than in gels made with unheated milk. The gels made with unheated milk had a coarser matrix which had much larger compartments (Kalab et al., 1983). The heat

treatment of the proteins results in interactions between the κ-casein and the whey proteins which in turn protect the casein micelles from aggregating. This is the reason for the heat step in the preparation of milk for yogurt production. However, as seen in control B, this is not enough to preserve the gel structure if it is disturbed prior to the completion of the acidification process. The cold step preserves the smaller micelles and prevents their aggregation. The effect of smaller casein micelles resulting in a stronger gel structure has also been demonstrated in studies involving the rennet induced coagulation of milk (Ford and Grandison, 1986, Niki and Arima, 1984). The effect of the chill step can be further seen in **Figure 5.2**. The disturbance of the weak gel structure had a noticeable effect on the Control-B samples, however all the agitation had no effect on the chilled samples. Shearing has been shown to have a negative effect on yogurt gel structure and stability, as the shearing of acidified milk without a cold step resulted in a decrease of WHC (Benezech and Maingonnat, 1993, Sodini et al., 2004). The samples that were chilled for 90 and 120 minutes had a larger volume and would result in a higher product yield since less whey would be lost.

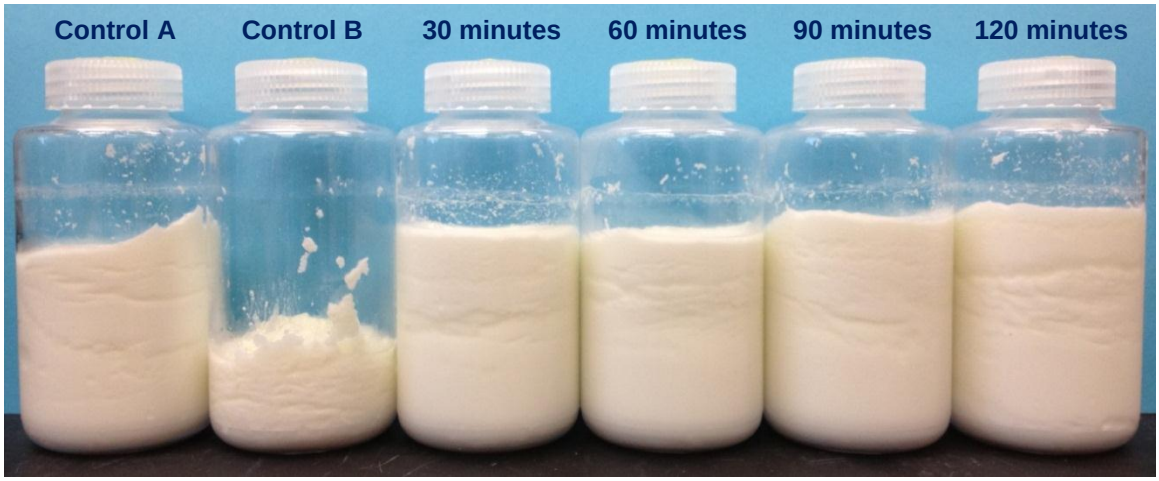

Figure 5.2. *Yogurt samples with whey removed after being centrifuged at 600 Xg for 15 minutes at 4 °C.*

Transmission Electron Microscopy

The TEM images of the gels can be seen in **Figure 5.3.** The yogurt samples that were fermented in their original individual containers (control A) showed small casein micelles (*ca*. 280.27 nm) that were loosely aggregated. The samples that were batch fermented until pH 5.2 and then poured into individual containers (control B) showed large casein micelles (*ca*. 414.76 nm) that were tightly aggregated. The samples that had a 90 min cold treatment prior to being poured into their individual container (90 min) showed similar results to the control A sample. The gel is made up of small casein micelles (*ca*. 271.57 nm) that were loosely aggregated. The TEM images demonstrate that with the inclusion of a cold step in the fermentation process of yogurt, it is possible to disturb the weak

gel structure being formed at pH values without any negative effects to the final yogurt gel matrix.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

β-casein was found in the supernatant of all the ultra-centrifuged (15 min at 100,000 x *g*) samples. As seen in **Figure 5.4**, the amount of β-casein found in the supernatant increased as the time the sample was kept at 5 ^{o}C increased.

Figure 5.5 shows the increase in the relative density of the β-casein bands in the gel. This is consistent with the previous observations of the effect of temperature and pH on the migration of β-casein out of the casein micelle.

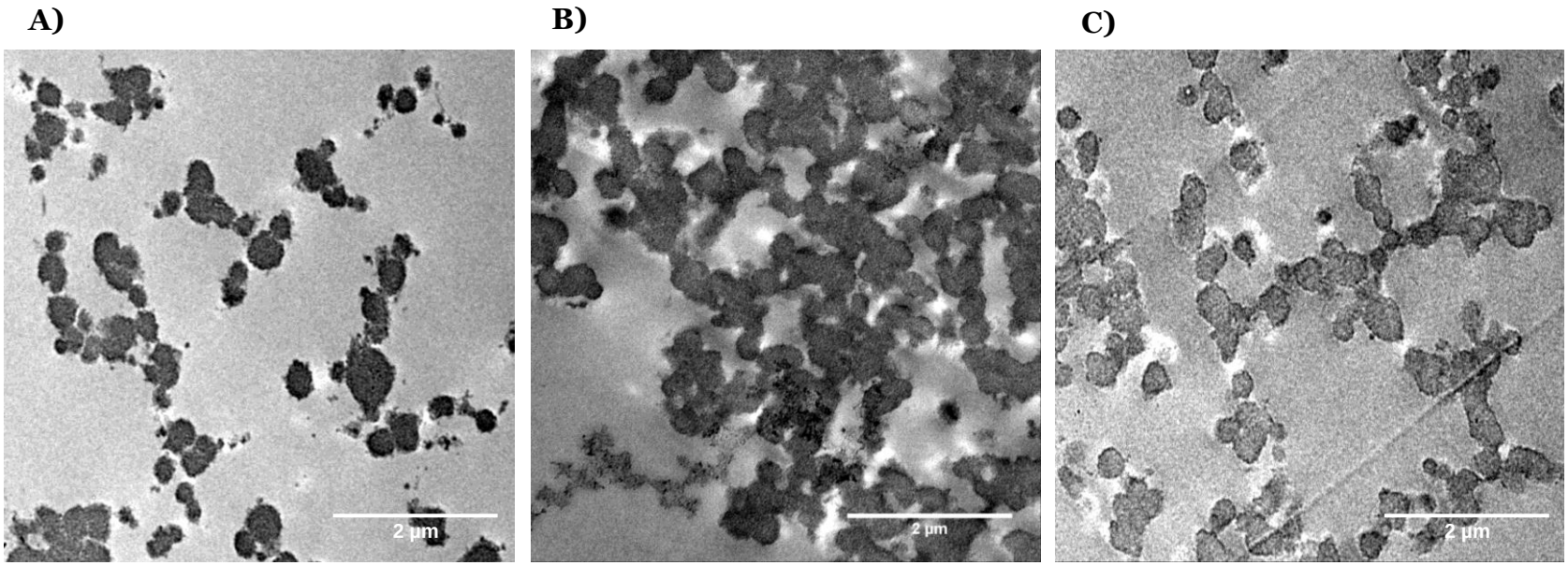

Figure 5.3. *Scanning Transmission Electron Microscopy (STEM) images of* ***A)*** *Yogurt fermented in container (Control A),* ***B)*** *Yogurt batch fermented to pH 5.2 and then poured into individual containers (Control B), and* ***C)*** *Yogurt made with acidified milk (pH 5.2) which was chilled for 90 min at 4 °C and then poured into individual containers. Scale bar = 200 nm*

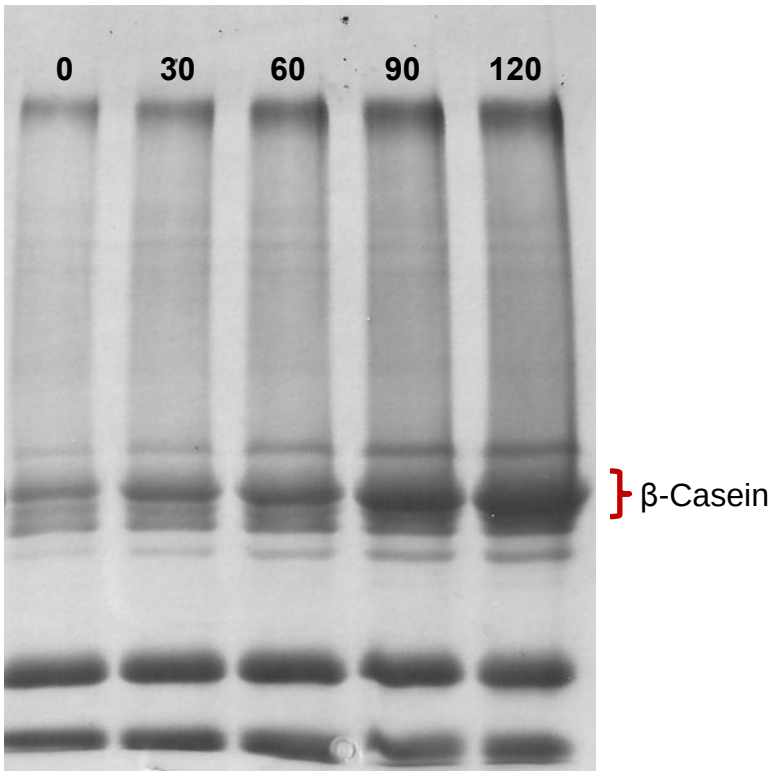

Figure 5.4. *SDS-15 % Polyacrylamide gel electrophoresis under reducing/denaturing conditions of the supernatant of ultra-centrifuged (100,000 x g, 15 min) acidified milk samples that were batch fermented at 40 °C until pH ~5.2 and held at 5 °C for 30, 60, 90, and 120 minute intervals.*

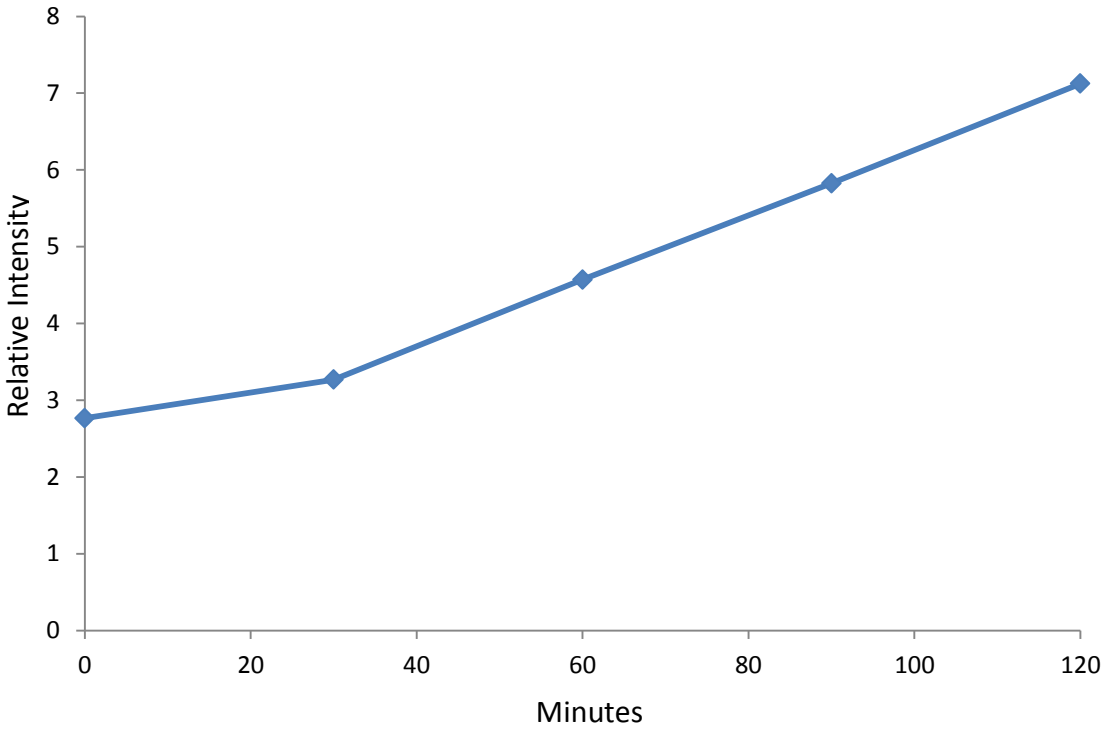

Figure 5.5. *Relative intensity of the of β-casein bands in the SDS- 15% Polyacrylamide gel electrophoresis under reducing/denaturing conditions of the supernatant of ultra-centrifuged (100,000 x* g*, 15 min) acidified milk samples that were batch fermented at 40 °C until pH ~5.2 and held at 5 °C for 30, 60, 90, and 120 minute intervals.*

Rheology

Figure 5.6 illustrates the changes in the viscosity of the sample from inoculation to a weak gel structure formation (pH ~5.2, 40 ^{o}C), and during different time intervals in the cold step (pH ~5.2, 5 ^{o}C) of the procedure. At the starting point, the inoculated milk had a viscosity (2.6 mPa.s) similar to the viscosity of milk (2.0 mPa.s). At a pH ~5.2, a weak gel structure had formed resulting in a time dependent non-Newtonian fluid as seen in **Figure 5.6**. Since the milk was pre-heated, the whey proteins associated with the casein micelles. Therefore, the presence of a weak gel structure at these pH values is consistent with the observations of Horne et al., in that the onset of gelation of pre-heated milk is close to the isoelectric point of the whey proteins (Horne and Davidson, 1993). During the cold treatment step, the viscosity of the samples returned to a more fluid like behavior although the samples had a higher viscosity (23.8 mPa.s) than the inoculated milk. This indicates that the weak gel structure was destroyed and the micelles returned to a form similar to the one found in fluid milk. The formation of a gel structure is inhibited at temperatures below 10 ^{o}C (Roefs et al., 1985, Roefs, 1987) so the acidified milk remains in liquid form for the duration of the cold treatment.

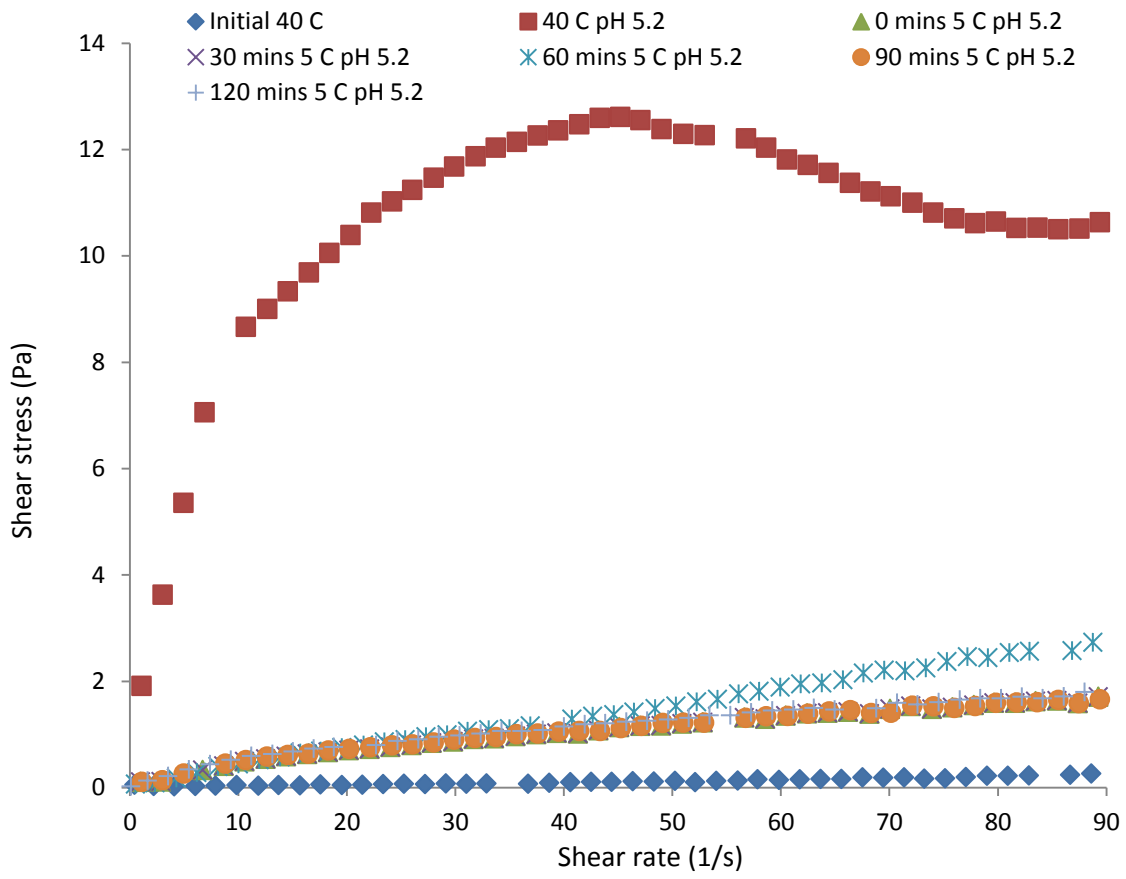

Figure 5.6. *Representative viscosity graph showing the changes in viscosity of: milk innoculated with yogurt cultures, acidified milk (pH 5.2 and 40 °C), and acidified milk held at 5 °C for 30, 60, 90, and 120 minute intervals.*

The G' of the final yogurt samples can be seen in **Figure 5.7**. As with the WHC, there was a statistical difference ($p < 0.05$) between the controls. And, there was a statistical difference between Control-B and the treatment samples.

A stronger yogurt gel structure has been achieved by other methods, such as fortifying the milk with different milk powders (e.g, skim milk powder, sodium caseinate, milk protein isolate, and micellar casein). Peng et al. demonstrated that yogurt fortified with sodium caseinate and micellar casein contain the highest casein to non-casein levels of all the fortified yogurts samples but had markedly different texture properties. This indicates that the textural properties of yogurt are influenced by other factors besides the ratio of casein to whey proteins (Peng et al., 2009). The most common method for producing stronger gels with better WHC is to increase the total solid content of the milk. The solid like behavior is usually increased by the addition of skim milk powder, as was done in our procedure. The increase in total solids results in higher interaction between the casein micelles. This interaction produced gels with a smaller pore size which increases the density of the gel matrix (Harwalkar and Kalab, 1986). By reducing the casein micelles dimensions, the addition to a cold step in the process would further assist in the production of a strong gel with a high WHC.

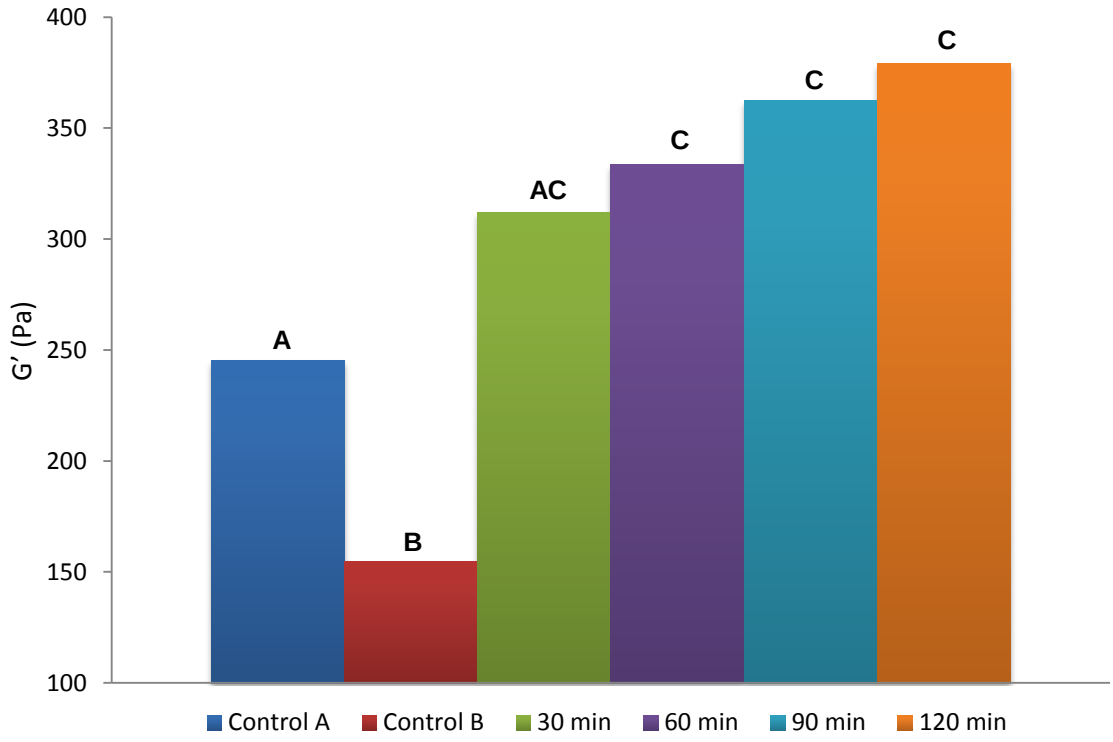

Figure 5.7. *Storage modulus (G') of yogurt samples that were batch fermented at 40 °C until pH ~5.2 and held at 5 °C for 30, 60, 90, and 120 minute intervals before being allowed to ferment to a pH of 4.6-4.5. Control-A was fermented in individual containers. Control-B was placed in containers at pH 5.2 and 40 °C without a cold step. Different letters indicate a statistical difference between the values ($p < 0.05$).*

The proposed mechanism for the modification of the casein micelle during the cold step can be seen in **Figure 5.8**. As the temperature and the pH decreases, β-casein and calcium migrate out of the casein micelle. Once the temperature is increased to the fermentation range, the liberated β-casein forms small micelles; which combined with the smaller original micelles form a thinner gel structure upon further acidification. The thinner structure results in a stronger gel with better WHC without the need for the addition of stabilizers or gums.

Conclusion

The pH and cold temperature induced migration of the β-casein and calcium from the casein micelle can be utilized in the manufacture of yogurt to improve on the properties of non-fat yogurt. By reducing the temperature of acidified milk at a pH ~ 5.2, during the fermentation product, it is possible to destabilize and reduce the size of the casein micelles due to the aforementioned migration of β-casein and colloidal calcium. These smaller micelles produce a firm gel with a high WHC. The introduction of a cold step allows for the reformation of a strong gel structure even after strong shear disturbs the weak gel network that forms at pH values of 5.3-5.2, this would permit the pumping and moving of yogurt without any negative effects on the final product when compared to yogurt set in the original containers. Furthermore, the addition of a cold step to the manufacture of yogurt would result in a stronger gel with good WHC without the need for the addition of stabilizers or gums.

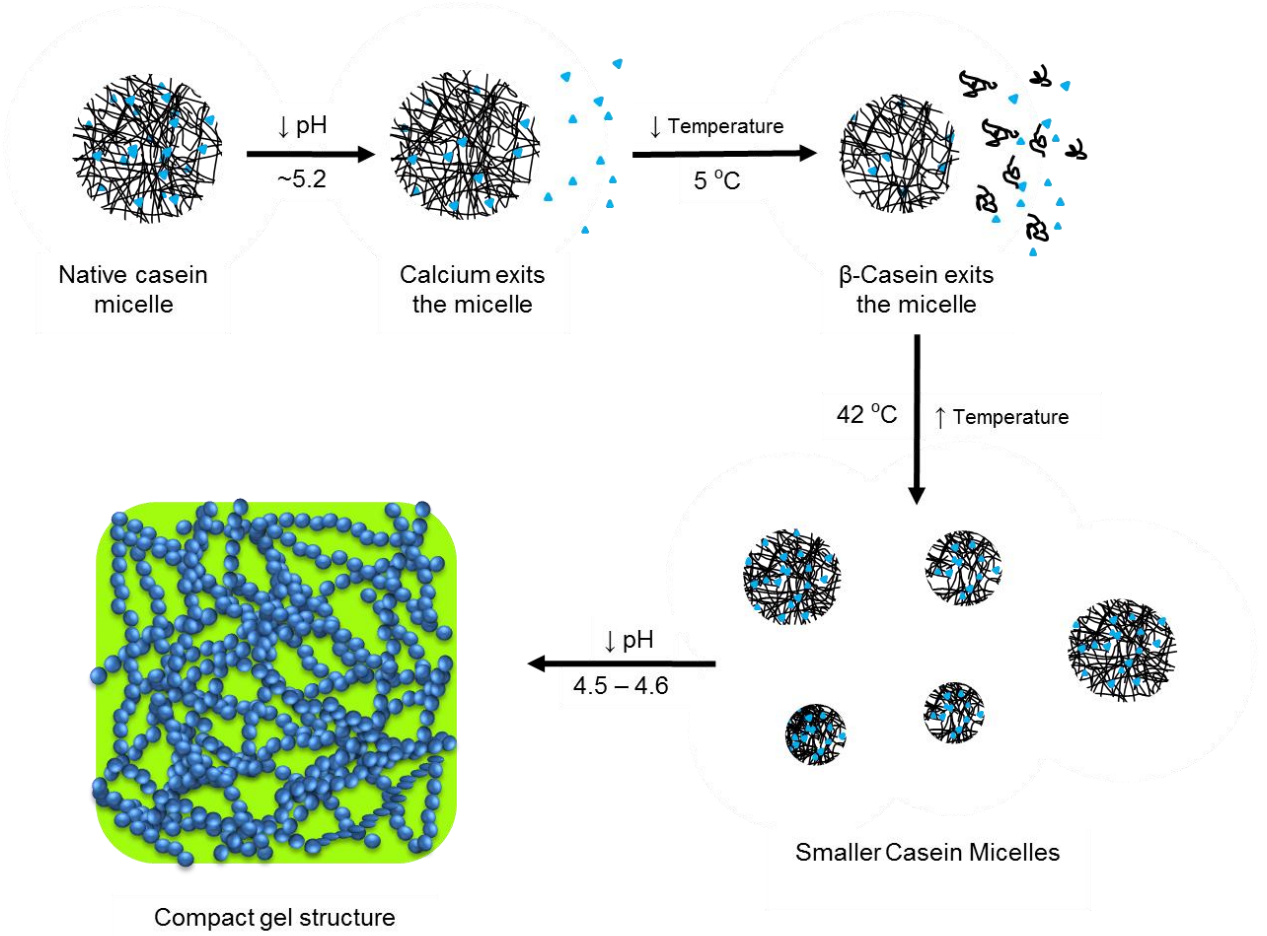

Figure 5.8. *Suggested mechanism for the changes to the casein micelles in acidified milk (pH ~ 5.2) held at 5 °C and their effect on the yogurt gel matrix.*

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CONCLUSION

A definitive structure of the native casein micelle structure continues to elude researchers. However, advances have been made towards this purpose. Data and images obtained via cryo-electron transmission electron microscopy allowed us to reconstruct a three dimensional model of the native casein micelle. This model demonstrated the presence of water filled cavities (*ca.* 20 to 30 nm diameter), channels (diameter larger than *ca.* 5 nm) and several hundred high density nanoclusters (6-12 nm diameter) within the interior of the micelles. No spherical protein submicellar structures were observed. Future work could focus on differentiating between the different proteins in the micelle. This would shed further light upon the nature of it structure.

These channels and cavities in the casein micelle allow for the native interaction of the micelles with the whey proteins. Whey proteins were found associated with isolated casein micelles regardless of the pH of the milk sample. However, we have no conclusive evidence that the introduction of a hydrophobic peptide into a raw skim milk solution did not result on the peptide being found associated with isolated casein micelles. The utilization of another analytical method, such as high pressure liquid chromatography, could provide definitive evidence of the interaction between the casein micelle and hydrophobic compounds.

A more detailed knowledge of the casein micelle structure and properties allow for the manipulation of the micelle to improve on its practical applications.

By taking advantage of the heat, ethanol, and pH mediated micelle disassociation, it was possible to improve the solubility of casein at pH values below 3.5 via esterification of the caseins. The esterification of casein results in a shift on their isoelectric point; which in turn results in a better solubility at low pH values. Further work in this field would involve a method for the positive confirmation of the esterification of the proteins as well as the effects of esterification to the safety of the proteins as a food ingredient.

Furthermore, by adding a chill step (3-5 ^{o}C/pH ~5.2) to the processing of non-fat yogurt, it was possible to improve the whey holding capacity of the product while also producing a firm product without the need for added stabilizers or gums. During the reduced temperature under acidified conditions of the chill step, β-casein and colloidal calcium phosphate move out of the micelles. This results in a decrease on the size of the original micelles. Once the temperature returns to the fermentation temperature of 47 ^{o}C, the β-casein self-associates into small micelles and together with the smaller original micelles forms a tight gel matrix. This more compact gel results in the firmer product and better resistance to syneresis.

Casein, as the principal protein in bovine milk, is fundamentally important to the dairy industry. By gaining an understanding of its structure and properties, it is possible to develop new applications and enhance their performance in dairy products.

VITA

Raymundo “Ray” Trejo was born in Brownsville Texas. He grew up in Guanajuato and Matamoros, Mexico. After high school graduation, he returned to the United States to pursue a degree in Computer Science at the University of Texas at Brownsville (UTB). While at UTB he became involved, by mere chance, with the Rancho del Cielo Biological Field Station Program. His experiences there led him to change his major to Biology. After obtaining his Bachelors of Science in Biology from UTB and marrying his college sweetheart, he enjoyed a rewarding career as a middle school Science teacher in Harlingen, TX and later in Nashville, TN. Serendipity once again intervened in his life, and he moved on to a position as a food inspector with the Nashville Davidson County Metro Health Department and later with the Knox County Health Department in Tennessee. While working as an inspector, he was bitten by the higher education bug, and decided to pursue a graduate degree in Food Science & Technology from The University of Tennessee. As a graduate student he was awarded the Graduate Diversity Enhancement Fellowship (2007), Access & Diversity Scholarship (2008), Joint Institute for Neutron Sciences Travel Fellowship (2010), First Place, IFT Dairy Division Manfred Kroger Research Paper Oral Competition (2011), and an honorable Mention, Ford Foundation Dissertation Fellowship (2012). He worked under the guidance and tutelage of Dr. Federico Harte. In the future, he plans pursue a career in the food industry, and to further continue to enjoy his life to the fullest in the company of his family, friends and numerous pets.